The Role of Androgens in Testicular Development and Dysgenesis

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Thesis submitted to the University of Edinburgh for the Degree of Doctor of Philosophy

September 2007

For my Grandad

Declaration

The studies undertaken in this thesis were the unaided work of the author, except where acknowledgement is made by reference. The work described in this thesis has not been previously accepted for, or is currently being submitted for another degree or qualification.

Hayley Scott
September 2007

Acknowledgements

M biggest ‘thank you’ goes to Richard Sharpe. I could not have wished for a better supervisor, and his patience, support, guidance and continued belief in my ability, has enabled me not only to produce this thesis, but to realise what I am capable of achieving when I ‘put it on the line’.

The ‘Sharpe group experience’ has been a fantastic one, and I have enjoyed every minute, so thank you to members, past and present, for being supportive, helpful and above all, great fun: Chris McKinnell, Marion Walker, Kim Mahood, Nina Hallmark, Gary Hutchison, Rod Mitchell, Michelle Welsh, Matt Jobling and Naomi Elkin.

For their technical advice and support I would like to thank the Histology team; Mike Millar (thank you also for all those lifts to work!), Sheila MacPherson, Arantza Esnal and Nancy Evans, whose RIA skills have also been greatly appreciated. Thanks also to Mark Fisken, for his expert help with the animals and treatments.

Thank you to Peter O’Shaughnessy, Karel De Gendt and Guido Verhoeven for the *tfm* and ARKO tissue, which has been invaluable to this thesis.

The Male Lab has been a terrific place to work, and has also provided me with some great friends: Many thanks to Gillian, Rob, Griet, Brigid, Karen, Nancy, George, Rowan, Carol, Vincent, Cathal and Lee.

The past three years have not been short of drama or the occasional emotional trauma, and without the following people, I’m not sure how I would have got through. So my deepest thanks go to some truly fantastic friends: Yvonne, Catriona, Sharon, Kim, Gary and Rod.

Huge thanks also go to my family; Denise, Graham, David and Eva, and close family friends; Chris, Rob and Sandra, who have always been there for me and whose supportive words and constant love has got me where I am today.

Last, but definitely not least, Andy, who has supported me through every step of this PhD, through the ups and the downs. My eternal thanks.

Hayley

2007

Abstract

Disorders of male reproductive health which manifest at birth (cryptorchidism, hypospadias) or in young adulthood (testicular germ cell cancer and low sperm counts), are common and may be increasing in incidence. These disorders have a common fetal origin and share risk factors; consequently they are hypothesized to comprise a testicular dysgenesis syndrome (TDS). TDS arises when maldevelopment (dysgenesis) of the fetal testis results in hormonal malfunctions and abnormal development and function of the somatic cells. It is thought that the suppressed intratesticular testosterone levels associated with TDS may account for subsequent low sperm counts, via a reduction in perinatal Sertoli cell proliferation/number. Sertoli cells do not express androgen receptors (AR) in fetal life in the human or rat, so it is hypothesised that any androgen effects on Sertoli cell number occur indirectly, via the AR positive peritubular myoid cells. Evidence from the di (*n*-butyl) phthalate (DBP)-treated rat model for TDS suggests that reduced androgen action may play a role in testicular dysgenesis as in patients with complete androgen insensitivity syndrome (CAIS; 'testicular feminization'), in whom focal areas of testicular dysgenesis have been reported.

The studies in this thesis sought to establish if reduced androgen levels/action in the fetal rat testis contribute to putative testicular dysgenetic features, namely reduced Sertoli cell number, occurrence of multinucleated gonocytes or abnormal aggregation of fetal Leydig cells, the precursor of focal dysgenesis. Pregnant rats were exposed to treatments or co-treatments expected to manipulate testicular testosterone levels (DBP, testosterone propionate; TP) or action (flutamide, DMBA) or to induce intrauterine

growth restriction (dexamethasone), another risk factor for TDS. The aforementioned endpoints were analysed in fetal testes and related to testicular testosterone levels and peripheral androgen action (anogenital distance). The same endpoints were evaluated in mice with inactivation of the androgen receptor (*tfm* or ARKO mice). As androgen action is assumed to be mediated indirectly, via the peritubular myoid cells, changes in peritubular myoid cell number and function were investigated in testes with suppressed androgens. *In vitro* studies were also used to investigate the role of androgens in Sertoli cell proliferation. Fetal rat testis explants were cultured with various chemicals designed to manipulate androgen action and Sertoli cell proliferation. Potential non-androgen related mechanisms of DBP action were investigated using Taqman RT-PCR to determine the mRNA expression of key developmental genes after exposure to DBP.

Sertoli cell number was reduced after exposure to treatments that reduced testicular testosterone levels, i.e. DBP alone or as a co-treatment, TP and dexamethasone. Sertoli cell numbers in ARKO mice were also significantly reduced. The occurrence of multinucleated gonocytes and large Leydig cell clusters were induced after exposure to DBP, alone or as a co-treatment, but not after exposure to TP or dexamethasone, and these dysgenetic endpoints did not occur either in *tfm* or ARKO mice. Rats exposed *in utero* to DBP have reduced testicular testosterone levels, however peritubular myoid cell number was unaffected by DBP, though AR expression in the peritubular myoid cells was delayed, and laminin and vimentin expression in Sertoli cells was altered after DBP exposure. DMRT-1 and DAX-1 mRNA expression levels were significantly reduced after DBP exposure, but this reduction was no longer evident once mRNA expression was corrected for Sertoli cell number.

In conclusion, these studies provide strong evidence that androgens play a role in regulation of Sertoli cell number/proliferation, and this is supported by a comparable reduction in Sertoli cell number in ARKO and *tfm* mice. However, since the treatments that reduce testicular testosterone in the rat, may also have a direct affect on the Sertoli cells, this alternate mechanism of action cannot be ruled out, and the administration of a treatment that reduces testicular testosterone without directly affecting Sertoli cells is required. These studies also show that reduced testicular testosterone levels are associated with multinucleated gonocyte formation and fetal Leydig cell aggregation, although this evidence it is not supported by parallel findings from the TP and dexamethasone exposed rats or the ARKO and *tfm* mice, as neither of these endpoints were identified as being affected in these animals. Aside from the delay in AR expression, there were no obvious changes in peritubular myoid cell number or the peritubular myoid cell markers examined in testes deprived of androgens, although there are other markers that could be investigated. mRNA analysis of the developmental genes investigated after DBP exposure, demonstrated no change in expression after correction for Sertoli cell number, suggesting that they do not play a role in the dysgenetic features observed in DBP exposed testes.

Presentations relating to this thesis

Poster presented at the 8th European Congress of Endocrinology, Glasgow, UK, 1st – 5th April 2006:

Androgen-dependent Sertoli cell proliferation as a target for environmental endocrine disruptors relevant to human male reproductive health

Oral presentation at the 14th European Testis Workshop, Bad Aibling, Germany, 22nd – 26th April, 2006:

Pathways of androgen regulation of Sertoli cell number in fetal life

Poster presented at the 5th Gordon Research Conference on Environmental Endocrine Disruptors, Il Ciocco, Italy, 4th – 9th July, 2006:

Androgen-dependent Sertoli cell proliferation as a target for environmental endocrine disruptors relevant to human male reproductive health

Poster presented at the 4th Copenhagen Workshop on Endocrine Disrupters, Copenhagen, Denmark, 28th - 31st May, 2007:

Androgens and Sertoli cell proliferation in the fetal testis: effects of exposure to di (*n*-butyl) phthalate ± dexamethasone-induced fetal growth restriction

Abbreviations

Abbreviation	Definition
µg	microgram
AF	activation function
AGD	anogenital distance
AHC	adrenal hypoplasia cogenita
AhR	aryl hydrocarbon receptor
AIS	androgen insensitivity syndrome
AMH	anti- Müllerian hormone
ANOVA	analysis of variance
AR	androgen receptor
ARE	androgen responsive elements
ARKO	androgen receptor knockout
Arx	aristaless-related homeobox gene
BrdU	bromo-2′-deoxyuridine
BSA	bovine serum albumin
CAIS	complete androgen insensitivity syndrome
cAMP	cyclic adenine mono-phosphate
CDKI	cyclin-dependent kinase inhibitors
CIS	carcinoma in-situ
CSL	cranial suspensory ligament
DAB	3,3 DiAminoBenzidine
DAX1	dosage sensitive sex reversal
DBP	Di (*n*-butyl) phthalate
DEHP	Di (2-ethylexyl) phthalate
Dhh	Desert hedgehog
DHT	dihydrotestosterone
DMBA	7,12-dimethylnez[a]anthracene
DMRT1	doublesex and Mab3 related transcription factor 1
DMSO	dimethyl sulphoxide
EGF	epidermal growth factor
e_m	embryonic day in the mouse/gestational day
e_r	embryonic day in the rat/gestational day
FGF9	fibroblast growth factor 9
FMR1	fragile x mental retardation
FSH	Follicle stimulating hormone
GCNA1	Germ cell nuclear antigen 1
gd	gestational day

Abbreviation	Definition
GnRH	gonadotrophin releasing hormone
GR	glucocorticoid receptor
hCG	human chorionic gonadotrophin
HDL	high-density lipoproteins
HGF	hepatocyte growth factor
Hpg	hypogonadal
HPG	Hypothalamic-Pituitary-Gonadal (axis)
HSD	hydroxysteroid dehydrogenase
hsp	heat shock protein
Insl3	insulin-like growth factor 3
IUGR	intrauterine growth restriction
kg	kilogram
L	litre
LH	Luteinising hormone
MEHP	mono-(2-ethylhexyl) phthalate
mg	milligram
MIS	Müllerian inhibiting hormone
MMP2	matrix metalloproteinase 2
MNG	multinucleated gonocyte
NADPH	nicotinamide adenine dinucleotide phosphate
NCAM	neural cell adhesion molecule
NGF	nerve growth factor
$P450_{C17}$	17α-hydroxylase/C17-20 lyase
$P450_{SCC}$	cholesterol side-chain cleavage
P75NTR	P75 neurotrophin receptor
PACAP-27	pituitary adenylate cyclase-activating polypeptide
PAH	polycyclic aromatic hydrocarbon
PAIS	partial androgen insensitivity syndrome
PAS	periodic acid-schiff
PDGF	platelet derived growth factor
PGD_2	prostaglandin D_2
PGK	phosphoglycerate kinase
PI	Proliferation Index
PTCH1	patched 1
RIA	radioimmunoassay
SCO	Sertoli cell only
SEM	standard error of the mean
Sf1	steroidogenic factor
SMA	smooth muscle actin

Abbreviation	Definition
SOX9	Sry related HMG box 9
SRB1	scavenger receptor class B type 1
Sry	sex determining region on the Y chromosome
StAR	steroidogenic acute regulatory protein
TBS	tris buffered saline
TDS	testicular dysgenesis syndrome
tfm	testicular feminised
TGF	transforming growth factor
TP	testosterone propionate
ts	tail somite
TSH	thyroid stimulating hormone
VIP	vasoactive intestinal peptide
WT1	Wilms tumour

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1 Literature Review

Disorders of male reproductive health which manifest at birth (cryptorchidism, hypospadias) or in young adulthood (testicular germ cell cancer and low sperm counts), are common and may be increasing in incidence (Sharpe and Skakkebaek, 2003). These disorders have a common fetal origin and share risk factors; consequently they are hypothesised to comprise a testicular dysgenesis syndrome (TDS). It is thought that TDS disorders arise when maldevelopment (dysgenesis) of the fetal testis results in abnormal development and function of the somatic cells (Sharpe and Skakkebaek, 2003; Skakkebaek et al., 2001), which might lead to altered hormone production by the Leydig cells or altered proliferation of one or more cell types such as the Sertoli cells.

In order to establish if reduced androgen levels/action contribute to testicular dysgenesis, it is first imperative to consider the dynamics of testis formation, the organisation of the various testicular cell types and the role of normal hormonal function.

1.1 Sex determination

The genetic sex of the embryo is determined at fertilisation, by the presence or absence of the Y chromosome, inherited from the father. The phenotypic sex however, is dependent on the development of secondary sexual characteristics, including external genitalia, and this hinges on whether the bipotential gonads develop as testes or ovaries. The majority of studies undertaken to elucidate the biology of sex determination and gonad development have been conducted in the mouse, so this is the model that will be referred to most frequently in this review. However, it is assumed

that events in the human and rat embryo follow a similar pattern, although there are differences in timing, and these will also be referred to where possible. Figure 1.1 summarises the temporal pattern of events that occur during human fetal testis development and Table 1.1 allows the comparison of the major events of testis development, between species.

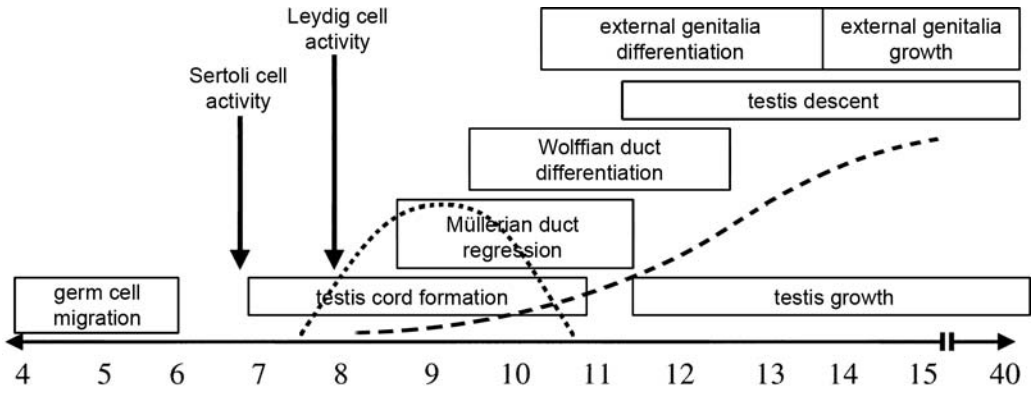

Figure 1.1 Temporal events in human fetal testis development during the 40 weeks of gestation. The dashed lines represent: (…) AMH and (---) testosterone secretion levels following Leydig cell differentiation at 8 weeks. Based on (Hughes, 2001; Voutilainen, 1992).

Table 1.1 Gestational ages at which each stage of testis development occurs in human, mice and rats. Taken from 'Sertoli cell biology' (Cupp and Skinner, 2005).

Species	Stage of testis development			
	Genital ridge	Bipotential gonad	Sry expression	Testis cord formation
Human (week of gestation/40 w)	5	6	7	7-8
Mouse (embryonic day)	9-10	10-11.5	10.5	11.5-12.5
Rat (embryonic day)	10-11	11.5-12.5	12	13.5-14

1.1.1 Emergence of the bipotential gonad

The bipotential gonads develop as paired structures within the developing urogenital system, which is derived from the intermediate mesoderm, and runs along the length of either side of the embryo. The urogenital system is divided into three temporal regions: the pronephros, which is vestigial in mammals; the mesonephros, from which the gonads develop and the metanephros, which becomes the kidney. The gonads develop from a single layer of coelomic epithelia, located on the ventrolateral surface of the mesonephroi. This 'mound' of coelomic epithelium (the genital ridge) then thickens and proliferates (Schmahl et al., 2000), giving rise to the gonads. In the mouse, the bipotential gonads are first visible around e_m10.5 - 11.5 (embryonic day in mouse; Capel, 2000).

1.1.2 Sry

Prior to the discovery of the Sry gene (sex determining region of the Y chromosome) in 1990 (Gubbay et al., 1990; Sinclair et al., 1990), it was believed that testis differentiation was induced by a diffusible testis-determining molecule, encoded for or regulated by the Y chromosome. It is now clear however, that the Sry gene encodes a DNA binding protein of the HMG-box family that regulates the expression of downstream genes (Ferrari et al., 1992; Rimini et al., 1995) and initiates the differentiation of the testes from the indifferent gonads, that would otherwise (in the absence of Sry) become ovaries (Gubbay et al., 1990; Sinclair et al., 1990). Transgenic experiments whereby XX mice carrying the Sry gene, develop as males (Koopman et al., 1991), and conversely, when Sry is deleted on the Y chromosome (Gubbay et al., 1992; Lovell-Badge and Robertson, 1990) or carry a mutation within the gene (Hawkins et al., 1992), ovary formation

ensues. These experiments confirmed that Sry is the only gene on the Y chromosome required to establish testis development.

Cells from the testes of chimaeric XX ↔ XY embryos were analysed, and ~90% of the Sertoli cells were shown to be XY, whereas all the other cell types were XX or XY with equal frequency (Palmer and Burgoyne, 1991). This indicated that the Sertoli cells are the only testicular cell type that requires the cell-autonomous expression of Sry (Palmer and Burgoyne, 1991). However, it also suggested, since ~10% of the Sertoli cells were XX, that Sry is not essential for the differentiation of all the Sertoli cells, and that other non-Sry-expressing cells can be recruited to the Sertoli cell fate (Palmer and Burgoyne, 1991). It has since been demonstrated using cell mixing experiments, that prostaglandin D_2 (PGD_2), which is produced and secreted by the Sertoli cells, recruits cells that do not express Sry and drives them to differentiate as Sertoli cells (Wilhelm et al., 2005). Other experiments have also suggested that the number of Sertoli cells within the gonad must reach a threshold level to ensure testis development, as chimaeric gonads containing fewer than 30% Sry expressing cells develop as ovaries (Palmer and Burgoyne, 1991). Consequently, the role of PGD_2 in recruiting non-Sry-expressing cells could be a backup mechanism to ensure that the threshold number of Sertoli cells is reached and the gonad develops as a testis.

Sry is expressed in the genital ridge from $e_m10.5$, its expression peaks at $e_m11.5$ and has disappeared by $e_m12.5$ (Hacker et al., 1995). Although Sry expression initiates the pre-Sertoli cells to differentiate into Sertoli cells (Albrecht and Eicher, 2001), this expression is transient, and so is not involved in the maintenance of Sertoli cell identity or function. It is consequently thought that Sry activates other genes to perform these roles.

Some of the genes located downstream of Sry include SOX9, DAX1 and FGF9, all of which are thought to play a part in Sertoli cell differentiation and function. SOX9 is thought to be the most likely candidate for a direct Sry target gene, and will be discussed in greater detail, along with DAX1 and FGF9, later in this chapter.

In addition to stimulating Sertoli cell differentiation from pre-Sertoli cells, Sry is also involved in the induction of testicular morphogenesis by inducing cell proliferation (Schmahl et al., 2000), and the migration of cells from the mesonephros into the gonad (Capel et al., 1999), which results in the formation of seminiferous cords. Presumably this effect occurs via effects on the Sertoli cells.

1.2 Testis formation and development

Testis formation is a complex process involving the migration, proliferation and differentiation of the various testicular cell types; the germ cells, Sertoli cells, peritubular myoid cells and Leydig cells, their organisation into seminiferous cords and the interstitial compartment, and the development of the testis specific vasculature. One of the first indications that the gonad has masculinised is its rapid increase in size (Schmahl et al., 2000). This is deemed to be the result of Sry-induced cell proliferation (Schmahl et al., 2000), and increased migration of cells from the coelomic epithelium (Karl and Capel, 1998), and the mesonephros into the testis (Capel et al., 1999; Martineau et al., 1997; Merchant-Larios and Moreno-Mendoza, 1998).

1.2.1 Origins of testicular cells

In mammals, the primordial germ cells do not arise within the gonad or the mesonephros, but instead migrate from the proximal epiblast of the

extraembryonic mesoderm, via the hindgut into the gonad (Adams and McLaren, 2002). This is one of the first events of gonad formation and will be discussed in greater detail later in this review. The somatic cells of the testis originate from either the coelomic epithelium or mesonephroi which lie adjacent to the gonad. The origins of the fetal Leydig cell still remain unclear and recent evidence has suggested that these cells do not have a single lineage origin. It is likely that both the coelomic epithelium and mesonephros contribute to the fetal Leydig cell population, the finer details of which will be discussed later in the chapter.

1.2.1.1 Coelomic epithelium

The coelomic epithelium is a single layer of cells that covers the entire coelomic cavity, including the morphological structures that become the gonad (Karl and Capel, 1998). Individual coelomic epithelial cells were labelled with the fluorescent dye DiI, and their fate was followed between tail somite (ts) 15 and 30 stages, which correspond to e_m11.2-12.5. The labelled coelomic epithelial cells proliferated and those cells that migrated to the gonad before ts 18, moved to the centre of the gonad and became Sertoli cells, confirmed by the co-expression of Anti-Mullerian hormone (AMH), a Sertoli cell-specific marker, as well as other cell lineages (Karl and Capel, 1998). The coelomic epithelial cells that did not migrate until after ts 18 remained outside the seminiferous cords and became part of the interstitial population (Karl and Capel, 1998).

At the same time that coelomic epithelial cells cease their migration into the testis, the basement membrane layer that lies beneath the coelomic epithelium becomes thick and continuous and forms the tunica albuginea, the capsule that surrounds the testis. It has been suggested that the formation

of the tunica albuginea creates a mechanical barrier that prevents the continued migration of the epithelial cells into the gonad. This does not explain however, why the migration of coelomic epithelial cells in XX gonads also terminates at this stage, as the XX gonad does not develop a tunica albuginea (Karl and Capel, 1998).

1.2.1.2 Mesonephros

As discussed earlier, the mesonephros constitutes part of the developing urogenital system and is derived from the embryonic mesoderm (Tilmann and Capel, 2002). The paired mesonephroi develop adjacent to the gonads, on either side of the gut mesentry. It has been demonstrated that cells from the mesonephros only migrate into the developing XY gonad (Martineau et al., 1997), and do so under the influence of Sry (Capel et al., 1999). These migrating cells go on to become the peritubular myoid cells, endothelial cells (blood vessels) and other uncharacterised interstitial cells (Buehr et al., 1993; Merchant-Larios et al., 1993).

The mesonephroi also comprise two ductal systems, the mesonephric (Wolffian) ducts and the paramesonephric (Müllerian) ducts. These ducts are ultimately sex specific and only one of the two systems persists, depending on the sex of the gonads and the supporting/inhibiting factors that they produce. In the fetal female, the Wolffian ducts regress and the Müllerian ducts persist, and conversely in the male, the Müllerian ducts regress and the Wolffian ducts persist. These processes constitute part of the sex differentiation of the embryo and will be discussed in greater detail later in the chapter.

1.2.2 Seminiferous cord formation

The formation of the seminiferous cords is a crucial process in the establishment of adult testis morphology. Testis cord formation occurs at e_m12.5 in the mouse (Karl and Capel, 1998) and at e_r13.5- 14 (embryonic day in rat) in the rat (Magre and Jost, 1984), and reminiscent of Sry expression, cord formation starts at the centre of the gonad and expands to the poles of the testis (Albrecht and Eicher, 2001). The testis cords are composed of clusters of germ cells encompassed by a layer of Sertoli cells and surrounded by a layer of peritubular myoid cells.

The first step in cord formation involves the aggregation of Sertoli cells around germ cells. In order for this aggregation to occur, it is essential that the Sertoli cells have differentiated, undergone a mesenchymal-to-epithelial cell transformation and polarized (Kanai et al., 1991; Kanai et al., 1992). The second step is dependent upon the migration of mesonephric cells from the adjacent mesonephros into the differentiating gonad.

Cell migration is a critical feature of testis cord formation. The cells that give rise to the peritubular myoid cells migrate in from the mesonephros into the developing gonad (Martineau et al., 1997), and surround the Sertoli cells. The Sertoli cells and peritubular myoid cells collaborate to induce the deposition of the basement membrane (Skinner et al., 1985; Tung, 1984; Tung and Fritz, 1987). The peritubular myoid cells have been shown to be essential to testis cord formation. In their absence, as shown in testis explant cultures lacking an adjacent mesonephroi, cord formation is disrupted (Buehr et al., 1993; Merchant-Larios et al., 1993). There also appears to be a critical time point, after which normal testis cord formation cannot occur. It has been demonstrated that if the migration of precursor cells into the gonad has not

commenced before e_m12.5 in the mouse, or e_r14 in the rat, then cord formation is disrupted (Tilmann and Capel, 2002). Mesonephric cell migration appears to be induced by growth factors expressed by the differentiating Sertoli cells. These factors include nerve growth factors (NGF), hepatocyte growth factors (HGF), platelet-derived growth factors (PDGF), fibroblast growth factors (FGF) and AMH (Brennan et al., 2003; Colvin et al., 2001; Cupp et al., 2000; Cupp et al., 2003; Ricci et al., 1999; Ross et al., 2003).

The germ cells are not involved in the initiation of cord formation, as testis cords still form in the absence of germ cells (McLaren, 1991; Merchant, 1975). Once the cells of the testis are organised into cords, the peritubular myoid cells and Sertoli cells collaborate to induce the deposition of the basement membrane.

1.2.2.1 Basement membrane formation

Once the peritubular myoid cells have surrounded the Sertoli cells in the process of cord formation, both cell types cooperate to deposit the basement membrane. The basement membrane is the interface between the epithelial Sertoli cells and mesenchymal peritubular myoid cells, and comprises three major structural components, the glycoproteins; collagen IV, that provides the structural framework for the basement membrane (Paulsson, 1992); laminin, a non-collagenous glycoprotein (Kleinman et al., 1993), and heparan sulphate proteoglycan (Hadley and Dym, 1987). Entactin also provides structural stability to the basement membrane by forming crosslinks between collagen IV and laminin (Chung and Durkin, 1990; Lian et al., 1992) and fibronectin can be found in the basement membrane of the peritubular myoid cells (Hadley and Dym, 1987). It is thought that the Sertoli cells produce

laminin and collagen IV (Borland et al., 1986; Davis et al., 1990; Skinner and Fritz, 1985b), as well as heparan sulphate proteoglycan (Borland et al., 1986) and entactin (Chung and Durkin, 1990), whilst the peritubular myoid cells are thought to synthesise fibronectin and collagen IV (Skinner et al., 1989; Skinner et al., 1985). Studies have shown that the basement membrane may play a role in supporting testis differentiation, and influence the peritubular myoid cells in particular (Tung and Fritz, 1991).

1.2.3 Testicular vasculature

The majority of cells that migrate into the gonad from the mesonephros are the vascular endothelial cells which form the testis-specific vasculature (Brennan et al., 2002; Martineau et al., 1997). These endothelial cells amalgamate to form the prominent coelomic vessel on the surface of the testis and the side branches in between the testis cords. It is thought that platelet-derived growth factor receptor-α (Pdgfr-α), and the Pdgf-A ligand, play a role in the formation of the testis vasculature, as Pdgfr-α $^{-/-}$ XY gonads demonstrate disruptions in the organisation of the vasculature (Brennan et al., 2003).

Once the vascular system is complete, blood flow is re-routed through the testis (Brennan et al., 2002). It is thought that the formation of the testicular vasculature may be important in providing oxygen to the rapidly growing testis and for the transport of testosterone, produced by the fetal Leydig cells. It has also been shown that vascular endothelial cells can produce regulatory signals involved in organ development (Lammert et al., 2001; Matsumoto et al., 2001), suggesting that the testis vasculature may play a role in influencing the development of the testis.

1.2.4 Sertoli cells

The Sertoli cells have two main functions; in fetal life they play a critical role in cord formation and secrete AMH (AMH will be discussed in section 1.3.1) (Mackay, 2000), and in postnatal life they provide the germ cells with the physical and metabolic support they need to undergo spermatogenesis (Sharpe, 1994).

As discussed earlier, the pre-Sertoli cells are derived from the coelomic epithelium and are prompted to differentiate into Sertoli cells by Sry. In addition to Sry, the Sertoli cells express a number of genes that play a role in testis development: SOX9, WT1, DAX1, FGF9 and DMRT1, which will be discussed in greater detail in this section. The Sertoli cells express many other genes such as Wnt-4, Lhx-1, Emx-1, Lhx-9 and Sf1, but to discuss these genes is beyond the scope of this review.

1.2.4.1 SOX9

SOX9 (Sry-related HMG box-9) expression occurs shortly after the onset of Sry expression, and in the same cell-type, the pre-Sertoli cells (Kent et al., 1996). SOX9 and Sry are both members of the SOX family of transcription factors. SOX9 is initially co-expressed with Sry in the nucleus of the pre-Sertoli cells (Lovell-Badge et al., 2002), but at e_m12.5, Sry expression diminishes and SOX9 expression remains confined to the Sertoli cells of the testis cords (Brennan and Capel, 2004), where it is maintained until after birth. This pattern of expression suggests that Sry activates the expression of SOX9, as well as pre-Sertoli cell differentiation, providing a marker for the latter. It has, however, been demonstrated that the ovaries of adult XX mice, mutant for oestrogen receptors α and β, contain abnormal cells that exhibit morphological features of Sertoli cells, including SOX9 expression. It is

therefore thought that these Sertoli cells transdifferentiate from ovary granulosa cells as a result of SOX9 expression, in the absence of Sry (Dupont et al., 2003). SOX9 is also expressed in a male-specific manner in many non-mammalian species that do not posses Sry. This evidence demonstrates that Sry cannot act as a transcription activator in these circumstances and suggests that there must be other means of activating SOX9 expression.

Mice with mutations in the fibroblast growth factor 9 (FGF9) signalling pathway demonstrate a loss of SOX9 expression, suggesting that FGF9 and extracellular signalling pathways play a role in SOX9 expression and primary sex determination (Colvin et al., 2001). Further to this, it is thought that FGF9 signalling acts through the FGF receptor 2 (FGFR2), which is expressed in the cell membrane of proliferating coelomic epithelial cells that become the Sertoli cells (Schmahl et al., 2004), and consequently express SOX9.

SOX9 plays a critical role in male sexual differentiation. Humans with SOX9 mutations suffer from severe dwarfism, a bone disorder known as campomelic dysplasia and XY sex reversal (Foster et al., 1994; Wagner et al., 1994). Mice with a null mutation for SOX9 are also subject to complete XY sex reversal (Barrionuevo et al., 2006; Chaboissier et al., 2004), and gain of SOX9 function in XX transgenic mice induces testis formation (Vidal et al., 2001). This evidence suggests that SOX9 plays a pivotal role in male sexual differentiation.

SOX9 has also been shown to act as a transcription factor and its up-regulation precedes the onset of AMH expression, leading to the hypothesis that SOX9 regulates AMH expression (Kent et al., 1996; Morais da Silva et al.,

1996). This hypothesis is supported by the demonstration that the human AMH promoter can bind SOX9 (De Santa Barbara et al., 1998).

1.2.4.2 WT1

Wilms tumour-associated gene (WT1) is expressed throughout the urogenital ridge, in the mesonephros, kidney and gonad (Armstrong et al., 1993). In kidney development, it is thought to mediate the outgrowth of the ureteric bud and the response of the metanephric mesenchyme to the ureteric bud (Moore et al., 1999).

WT1 encodes a nuclear zinc-finger protein that can function as a transcriptional activator and repressor. It is a complex gene and has a complex locus, encoding for 24 known variations of the WT1 protein (Hohenstein and Hastie, 2006). WT1 mutations in humans can result in mild genitourinary malformations that predispose to childhood kidney tumours, or Denys-Drash syndrome and Frasier syndrome, which both result in dysgenetic testes (Jaubert et al., 2003), as well as urogenital malformations. In both of these syndromes the phenotype is always more severe in XY patients than in XX patients (Hastie, 1992), suggesting that WT1 has a testis-specific role in addition to its role in early gonadal development in both sexes. Mice with a heterozygous deletion for WT1 do not develop tumours or exhibit genitourinary malformations, but their gonads are absent and they have arrested kidney development (Kreidberg et al., 1993).

It has been demonstrated that WT1 can activate the DAX1 promoter (Kim et al., 1999). Humans with mutations in DAX1, have the X-linked clinical syndrome adrenal hypoplasia congenita (AHC; (Muscatelli et al., 1994), which usually gives rise to a shortage of male sex hormones and results in under-developed reproductive tract tissues (hypogonadotrophic

hypogonadism) and cryptorchidism. Although it is not clear where DAX1 is expressed, it has been reported to be localised to the fetal mouse Sertoli cell (Meeks et al., 2003), fetal rat Leydig cell cytoplasm (Majdic and Saunders, 1996) and fetal mouse Leydig cells (Ikeda et al., 1996). DAX1 deficient mice exhibit poorly differentiated Sertoli cells, Leydig cell hyperplasia (Jeffs et al., 2001), a reduction in peritubular myoid cell number and incomplete cord formation (Meeks et al., 2003), which also suggests an important role in testis development and differentiation.

1.2.4.3 DMRT1

In 1998, a human gene was discovered with homology to the *Drosophila* sex regulatory gene *doublesex*, and the *C. elegans* sex regulator *mab-3* (Raymond et al., 1998). This gene, doublesex and mab3 related transcript (DMRT1) is a member of the DM family, which has been shown to be involved in sex differentiation in organisms such as *Caenorhabditis elegans* (*C. elegans*), *Drosophila*, fish and mammals (Raymond et al., 2000).

In mice, DMRT1 is initially expressed in the indifferent gonads of both sexes, from e_m10.5, and as is the case with chickens, turtles and humans, higher levels of expression are maintained in the testes than in the ovaries (Kettlewell et al., 2000; Moniot et al., 2000; Raymond et al., 2000). After e_m12.5 in the mouse, expression of DMRT1 is restricted to the seminiferous cords, specifically in the Sertoli cells and germ cells (Raymond et al., 1999; Raymond et al., 2000).

The human gene DMRT1, maps to a region of chromosome 9p and upon deletion, XY gonadal dysgenesis ensues (Veitia et al., 1998). In the mouse null mutant, however, the phenotype is not as severe and does not affect embryonic gonadogenesis, although postnatal germ cells are lost (Raymond

et al., 2000) and Sertoli cells do not mature (Kim et al., 2007). This suggests that either DMRT1 is not important for sex determination, or that other factors can compensate for its loss in the mouse.

1.2.4.4 FGF9

FGF9 is initially expressed in the indifferent gonads of both sexes from e_m11.5 (Schmahl et al., 2004). However, after the onset of Sry expression, it becomes male gonad specific and is expressed by the fetal Sertoli cells (Kim et al., 2006). In addition to its role in the activation of SOX9 expression, it is also said to play a role in Sertoli cell proliferation, organisation and differentiation (Adams and McLaren, 2002). In mice with null mutations for FGF9, XY gonads develop as ovaries and there is a disruption of cell proliferation, mesonephric cell migration, testis cord formation and Sertoli cell differentiation (Colvin et al., 2001). It has been suggested that the reduced proliferation of coelomic epithelial cells, the precursor Sertoli cells, could mean that the critical threshold number of Sertoli cells is not met and may be responsible for the testis developing as an ovary (Schmahl et al., 2004). Conversely, the addition of FGF9 to cultures has been shown to increase Sertoli cell proliferation (Willerton et al., 2004).

1.2.4.5 Sertoli cell proliferation

Proliferation of the Sertoli cell population is important because, in adulthood, each Sertoli cell can only support a finite number of germ cells through spermatogenesis. Consequently, the number of Sertoli cells per testis determines both the testis size and maximum number of sperm that it can produce (Orth, 1982; Sharpe et al., 2003). After puberty, the Sertoli cells are no longer mitotic, so the population at that point, is definitive.

The Sertoli cells proliferate during two periods of life: fetal or neonatal and peripubertal, although this is obscured in species such as the rat, in which neonatal and peripubertal periods overlap (Sharpe et al., 2003). In rats, the period of greatest Sertoli cell proliferation occurs between e_r18 and the day of birth (Levine et al., 2000). Sertoli cell proliferation then declines during the first 2-3 weeks after birth (Orth, 1982).

Follicle stimulating hormone (FSH) has been shown to stimulate Sertoli cell proliferation in both fetal (Orth, 1984) and postnatal life (Griswold et al., 1977; Orth and Boehm, 1990). In the absence of FSH, after the decapitation of e_r18 rats or after administration of an FSH antibody, Sertoli cell number was clearly reduced (Orth, 1984). However, the FSH receptor is not expressed in the fetal testis until $e_r18.5$ (Rouiller-Fabre et al., 2003) and FSH is only first detectable at e19.5 (Majdic et al., 1997). An investigation into Sertoli cell number in fetal and postnatal hypogonadal (*hpg*) mice, which have no GnRH and therefore lack circulating gonadotrophins (FSH and Luteinising hormone; LH), demonstrated that Sertoli cell number was normal throughout fetal life, but was reduced by 30% on the day of birth, compared to normal mice. Although Sertoli cells proliferated for longer in the *hpg* mice, proliferation rates were lower, resulting in a 50% reduction in Sertoli cell number on postnatal day 20. This suggested that fetal Sertoli cell proliferation is independent of gonadotrophins, whereas postnatal Sertoli cell proliferation requires gonadotrophins (Baker and O'Shaughnessy, 2001).

Further to these findings, recent studies using three transgenic/mutant mouse lines (FSH knockout, FSH receptor knockout and testicular feminised male mice; *tfm*) revealed that only the *tfm* mice had reduced Sertoli cell numbers at birth, suggesting that androgen action rather than FSH, plays an

important role in Sertoli cell proliferation in the fetal testis (Johnston et al., 2004).

In addition to hormones, epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) have also been shown to stimulate postnatal Sertoli cell proliferation, at least in culture (Petersen et al., 2001).

Whereas FSH and androgens have been shown to have a stimulatory effect on Sertoli cell proliferation, thyroid hormone has been shown to inhibit Sertoli cell proliferation in rodents. It has been shown that hypothyroidism extends Sertoli cell proliferation (Van Haaster et al., 1992), whereas hyperthyroidism shortens the period of Sertoli cell proliferation (van Haaster et al., 1993). Thyroid hormone impedes Sertoli cell proliferation by increasing the expression of the cyclin-dependent kinase inhibitors (CDKIs), $P27^{kip}$ and $P21^{cip}$, which inhibit the progression of the Sertoli cell through the cell cycle and as such bring about premature maturation (Buzzard et al., 2003b; Holsberger et al., 2003).

1.2.4.6 Sertoli cell differentiation

Sertoli cells differentiate, or functionally mature, around the onset of puberty (Sharpe et al., 2003), at the same time that they exit the cell cycle (Gondos and Berndston, 1993). They undergo morphological changes; the nucleus enlarges and becomes tripartite and the nucleolus becomes more distinct (Sharpe et al., 2003) and between each Sertoli cell the formation of specialised tight junctions creates the blood-testis barrier (Gondos and Berndston, 1993).

1.2.5 Peritubular myoid cells

The peritubular myoid cells are flat, smooth-muscle-like cells that, as discussed earlier, migrate into the gonad from the mesonephros and

contribute to testis cord formation by surrounding the clusters of Sertoli cell-enclosed germ cells, and collaborating with the Sertoli cells to deposit the basement membrane (Buehr et al., 1993; Martineau et al., 1997). The peritubular myoid cells are the only cells in the testis that do not have a counterpart cell in the ovary. It has been suggested that this is due to the fact that cells only migrate from the mesonephros into the XY gonad after the expression of Sry (Capel et al., 1999; Martineau et al., 1997).

The peritubular myoid cells proliferate during fetal life, but proliferation declines rapidly after birth and is minimal after three weeks of age (Palombi et al., 1992). The age at which the peritubular myoid cells differentiate is less clear. It has been reported that the peritubular myoid cells do not differentiate until the early stages of puberty, when they become flatter and longer (Skinner, 1991), but a more recent publication demonstrated that peritubular myoid cells express all known markers of differentiation; desmin, α-smooth muscle isoactin (SMA) and alkaline phosphatase, shortly after birth (Palombi et al., 1992). SMA expression has since been reported in the peritubular myoid cells of e_r19 fetal rat testes (Fisher et al., 2003).

Knowledge of the peritubular myoid cells is limited, as there is still no cell specific marker available (Jeanes et al., 2005). The peritubular myoid cells, along with the Leydig cells, do however express the Patched 1 (PTCH1) receptor for Desert hedgehog (Dhh), a factor secreted by the Sertoli cells (Bitgood et al., 1996). It has been suggested that Dhh/PTCH1 signalling between the Sertoli cells and peritubular myoid cells may play a role in peritubular myoid cell migration and differentiation, since inhibition of the Dhh/PTCH1 signalling pathway results in disrupted cord formation (Yao

and Capel, 2002) and mice with a null mutation for Dhh have impaired differentiation of the peritubular myoid cells (Clark et al., 2000).

The peritubular myoid cells have also been shown to express the androgen receptor during fetal and postnatal life (You and Sar, 1998), and it has been suggested that as a result they are likely to play a significant role in testicular development (Anderson et al., 2002).

1.2.6 Primordial germ cells

The primordial germ cells are the embryonic precursors of the gametes and as such, are responsible for the passing on of genetic information between generations. As was briefly discussed earlier, the primordial germ cells do not arise from the coelomic epithelium or mesonephros, as the somatic cells of the testis do, but instead arise from a small population of epiblast cells in the extraembryonic mesoderm, near the base of the allantois (a membranous sac that develops from the posterior section of the gut in mammalian embryos), where they are first detected at e_m7.5 (Ginsburg et al., 1990). The primordial germ cells then migrate to the dorsal mesentry, via the hindgut, before colonising the genital ridge, a process that is complete by e_m12.5. The primordial germ cells were thought to migrate to the genital ridge independently (De Felici and Dolci, 1987), but it has since been demonstrated that they associate with one another via cytoplasmic processes called filopodia, which form extensive networks interlinking the cells (Bendel-Stenzel et al., 2000). It has been demonstrated that the cadherins, a family of cell adhesion molecules, are involved in the association of the primordial germ cells and in the absence of E-cadherin, there is an increase in the number of ectopic primordial germ cells (Bendel-Stenzel et al., 2000).

During their migration to the genital ridge, the primordial germ cells proliferate rapidly, and by the time their proliferation is complete, at e_m13.5, there are over 25,000 gonocytes in the gonad (Tam and Snow, 1981). It has been demonstrated that this proliferation is autonomously programmed, as primordial germ cells that accidentally migrate into the adrenal, are seen to cease proliferation at the same time as those primordial germ cells that migrate into the gonads (Richards et al., 1999).

Once the primordial germ cells have completed their migration and arrived at the genital ridge, they mature into fetal germ cells (gonocytes) and express GCNA1 protein (germ cell nuclear antigen-1), a marker of post-migratory germ cells (Enders and May, 1994). What triggers the transition from primordial germ cell to gonocyte is still unclear. Although the primordial germ cells appear to develop into gonocytes post-migration, and upon exposure to the genital ridge, *in vitro* cultures of primordial germ cells have shown that this exposure is not essential for their maturation (Richards et al., 1999).

The gonocytes are bipotent cells, and whether they become meiotic oocytes or mitotic prospermatogonia, is dependent upon the sex of the somatic cells of the gonad. This was demonstrated when XY primordial germ cells developed into oocytes in the female embryo and XX primordial germ cells developed into prospermatogonia in the male embryo (Ford et al., 1975; Palmer and Burgoyne, 1991).

Primordial germ cells that migrate into ectopic tissue, such as the adrenal cortex, enter meiosis, and do so at a similar time to those primordial germ cells that migrated successfully into the female gonad. This suggests that entry into meiosis is a cell-autonomous programme based on an intrinsic

clock (McLaren and Southee, 1997). It also implies that the embryonic testis produces a factor that blocks entry into meiosis and instead initiates mitotic arrest, therefore establishing a spermatogenic fate (Dolci and De Felici, 1990; McLaren and Southee, 1997). Primordial germ cells can be rescued from this signal if they are removed from the XY gonad before e_m11.5, and they develop as oocytes in lung aggregates. By e_m12.5, however, the primordial germ cells are committed to spermatogenesis (McLaren and Southee, 1997). The current evidence suggests that retinoic acid, produced by the mesonephros in both sexes, induces germ cells to enter meiosis (Bowles et al., 2006). The male germ cells are protected from retinoic acid by the Sertoli cells, which express CYP26B1, an enzyme that catabolises retinoic acid and therefore acts as a male-specific meiosis-inhibiting factor (Bowles et al., 2006).

In the mouse, the male germ cells remain mitotically arrested in G1/G0 until a few days after birth, when they resume proliferation (Hilscher et al., 1974; McLaren, 1984). In the rat, the male gonocytes also remain in mitotic arrest until just after birth. They then migrate from the centre of the cord to the basement membrane and begin to differentiate into spermatogonia (Boulogne et al., 1999).

1.2.7 Leydig cells

The Leydig cells were first described by Franz Leydig in 1850, but the endocrine role of the Leydig cell was not established until 1903. In the 1930's it was demonstrated that the product of the Leydig cells was a steroid and was named testosterone, and in the 1950-60's the Leydig cells were identified as the main source of testiscular androgens in rodents and man.

There are two types of Leydig cell, both of which are responsible for steroidogenesis; the fetal Leydig cells and adult Leydig cells, which develop

as distinct lineages (Habert et al., 2001; Mendis-Handagama and Ariyaratne, 2001), and occur simultaneously in the testis, suggesting that they are derived from separate cell lines and are developmentally unrelated (Kerr and Knell, 1988). The structural features of the adult and fetal Leydig cells are similar, although their functional properties differ considerably (Huhtaniemi, 1989).

The adult Leydig cells differentiate after birth, and are thought to arise from undifferentiated precursor cells from the mesenchymal cells of the interstitium (De Kretser and Kerr, 1994; Hardy et al., 1993). Further discussion of the adult Leydig cell population is out of the scope of this review, as the studies in this thesis concentrate on fetal life.

The role of the fetal Leydig cells is to produce androgens, and thus ensure the masculinisation of the embryo. They also secrete insulin-like growth factor 3 (Insl3), which, combined with androgen action, induces testicular descent (Klonisch et al., 2004). These processes will all be discussed in greater detail in section 1.3.

1.2.7.1 Fetal Leydig cells

The fetal Leydig cells first appear in the interstitial tissue of the testis at $e_m12.5$, differentiating from mesenchyme-like stem cells (Byskov, 1986). The origins of the fetal Leydig cells still remain uncertain, although evidence suggests that both the coelomic epithelium and mesonephros may give rise to the fetal Leydig cell population.

The coelomic epithelium is known to give rise to the Sertoli cells and a significant number of interstitial cells (Karl and Capel, 1998). However, examination of the coelomic epithelium during its proliferative stage, using steroidogenic factor 1 (Sf1; a factor essential for Leydig cell differentiation;

Luo et al., 1994) as a marker for Leydig cells, revealed that relatively few of the coelomic epithelial cells differentiated into Leydig cells (Yao et al., 2002). Consequently, it has been reported that although some Leydig cells may differentiate from the coelomic epithelium, this is not their main point of origin (Brennan et al., 2003).

Mesonephric cells migrate into the developing testis at around e_m11.5, (Buehr et al., 1993) and it has been suggested that some of these mesonephric-derived cells may differentiate into fetal Leydig cells (Merchant-Larios and Moreno-Mendoza, 1998; Nishino et al., 2001). A study culturing the developing mouse testis in the absence of the mesonephros at e_m11.5 demonstrated the presence of a normal Leydig cell population, suggesting that if the mesonephros does contribute to the Leydig cell population, then these cells must migrate into the testis before e_m11.5 (Merchant-Larios et al., 1993). It has also been suggested that the neural crest may contribute to the fetal Leydig cell population, as these cells express the neural cell adhesion molecule (NCAM; Mayerhofer et al., 1996). However, no further evidence was found to show that neural crest cells differentiate into fetal Leydig cells (Brennan et al., 2003).

Fetal Leydig cell development and differentiation requires Sf1 expression, and the Sertoli cells also play a role by providing Dhh and Pdgf-α. These genes/factors will be discussed in more detail below. In addition, the peritubular myoid cells, endothelial cells and interstitial fibroblasts are also involved in Leydig cell differentiation, expressing the X-linked aristaless-related homeobox gene (Arx), which has been shown to be necessary for Leydig cell development (Kitamura et al., 2002).

In the rat, the fetal Leydig cells begin to synthesise testosterone at e15.5, and this functional maturation occurs at the same time as fetal Leydig differentiation (Warren et al., 1973). The fetal Leydig cells are very active and steroid production per cell is much greater than in the adult Leydig cells (Huhtaniemi et al., 1982; Tapanainen et al., 1984).

Adult Leydig cell steroidogenesis is regulated by LH, part of the HPG-axis, and this will be discussed later in the chapter. Although LH is detected in the fetal testes from e_r17.5 at low levels (El-Gehani et al., 1998b), it has been demonstrated that fetal Leydig cell function is normal in the absence of LH, in *hpg* mice, (O'Shaughnessy et al., 1998) or the LH receptor (Zhang et al., 2004). Perinatal regulation of steroidogenesis does, however, require LH (O'Shaughnessy et al., 1998), implying that there must be a switch, at around the time of birth, from gonadotrophin independent to gonadotrophin dependent steroidogenesis (O'Shaughnessy et al., 1998).

Once the fetal Leydig cells have differentiated they proliferate, the peak of which occurs between e_r18-19 (Ziegler et al., 1983), but after birth fetal Leydig cell numbers regress, although some fetal type Leydig cells remain in adult life (Kerr and Knell, 1988).

1.2.7.1.1 Sf1

Sf1 is an orphan nuclear receptor that regulates the expression of the steroidogenic enzymes (details of which will be discussed later) required for hormone synthesis, embryonic formation of the gonads and adrenal glands (Hatano et al., 1994; Luo et al., 1994; Parker and Schimmer, 1994). Sf1 also targets the Sertoli cell and regulates AMH expression (Hatano et al., 1994).

The occurrence of two Sf1 positive cell populations in the adrenal gland and the gonad suggests that these cells share a common origin (Hatano et al.,

1996). In fact Sf1 positive cells can be identified at the cranial end of the mesonephros at e11.5 (Hatano et al., 1996), and these cells are then reported to separate into two cell populations, one in the region of the adrenal cortex and the other near the coelomic epithelium, where the gonad develops (Hatano et al., 1996).

Targeted disruption of Sf1 expression in mice resulted in regression of the adrenal gland and gonad as a consequence of increased apoptosis, although prior to this regression, the primordial germ cells were identifiable in the gonadal ridge, suggesting that Sf1 expression is required for the development of the somatic cell lines (Luo et al., 1994).

1.2.7.1.2 Dhh

Desert hedgehog (Dhh) is secreted by the Sertoli cells (Bitgood et al., 1996), and its receptor, Patched 1 (PTCH1), is expressed in Leydig cells and peritubular myoid cells (Clark et al., 2000). Inhibition of the Dhh/PTCH1 signalling pathway results in disrupted cord formation (Yao and Capel, 2002), incomplete Leydig cell differentiation (Yao et al., 2002) and in mice lacking Dhh, 92.5% of the males were under-masculinised, with abnormal peritubular myoid cells and severely restricted spermatogenesis (Clark et al., 2000). This implies that Dhh signalling is essential for fetal Leydig cell development and cord formation, as well as for the formation of adult Leydig cells.

1.2.7.1.3 PDGFR-α

The importance of platelet derived growth factor receptor-α (Pdgfr-α) in the development of the testicular vasculature, cord formation and Leydig cell differentiation was demonstrated using Pdgfr-α knockout mice (Brennan et

al., 2003), and the authors concluded that Pdgfr-α was necessary for the differentiation or expansion of the fetal Leydig cells, along with two of its three ligands; Pdgf-A and Pdgf-C (Brennan et al., 2003). This study also concluded that Pdgfr-α may function in parallel with Dhh to promote the expansion and/or differentiation of the fetal Leydig cells (Brennan et al., 2003).

1.3 Masculinisation and the role of hormones

Becoming a male requires continued intervention to modify the female set-up programme (Hughes, 2001), so once the fetal testis has formed, masculinisation of the embryo and further development of the testis is driven by three hormones produced by the fetal testis: AMH, Insl3 and testosterone. The roles of these hormones are summarised in Figure 1.2.

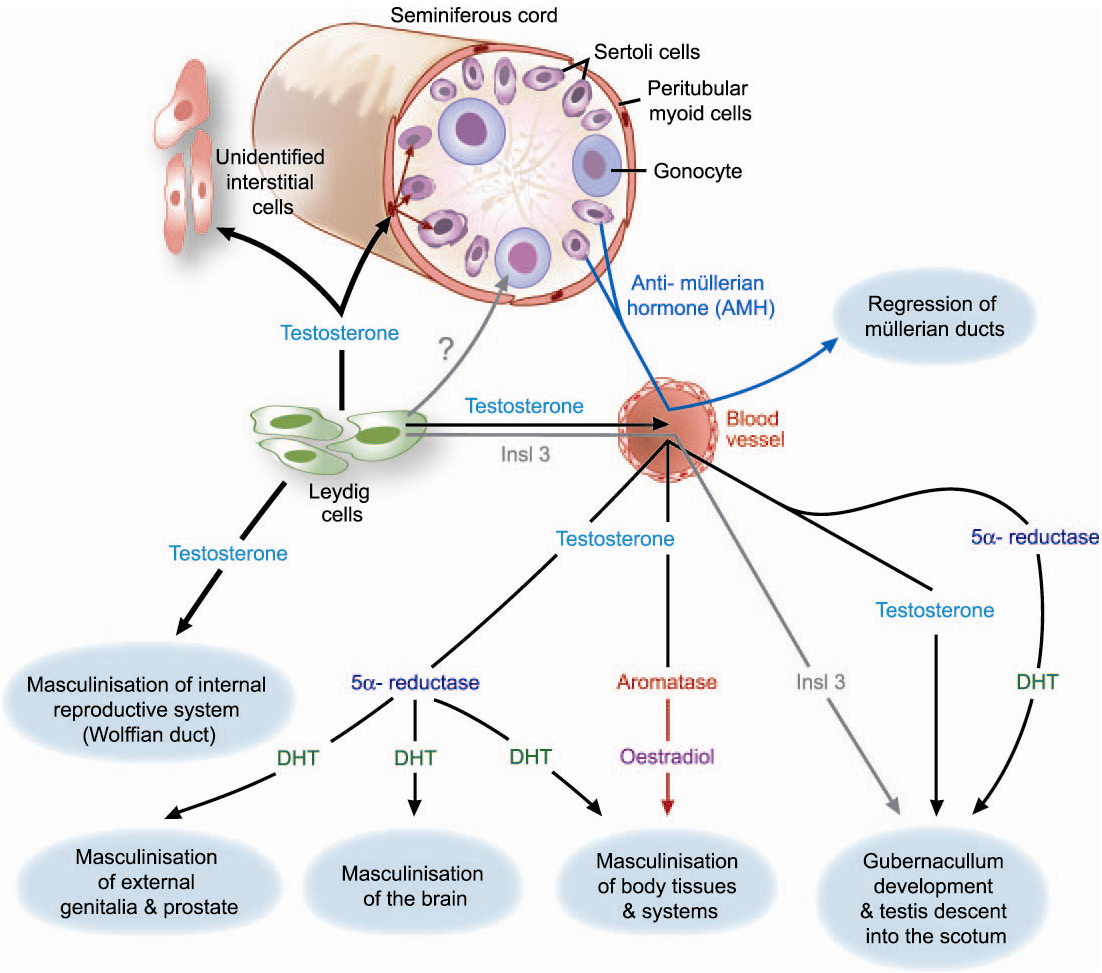

Figure 1.2 This diagram illustrates the site of production and the target tissues of the three hormones; AMH, Insl3 and testosterone, responsible for masculinisation in the fetal male. The enzymes 5α-reductase and aromatase are expressed in the peripheral target tissues. Figure reproduced from (Sharpe, 2006).

1.3.1 AMH

AMH, also known as Müllerian-inhibiting substance (MIS), is a glycoprotein belonging to the transforming growth factor-β (TGF-β) family, which also includes inhibin and activin, and is produced by the fetal Sertoli cells (Lane and Donahoe, 1998). As discussed previously in section 1.2.1.2, both male and female embryos initially have two pairs of genital ducts and the role of AMH, in male sexual differentiation, is to initiate the regression of the Müllerian ducts, which in the female, differentiate into the upper vagina, uterus and oviducts. In order for Müllerian duct regression to occur, AMH

must bind to its receptor AMHR2 (also known as MISRII), on the surface of the Müllerian duct mesenchymal cells, which induces a signalling cascade that results in the production of matrix metalloproteinase 2 (MMP2), which stimulates apoptosis in the Müllerian duct epithelial cells (Roberts et al., 2002). This process of Müllerian duct regression occurs between e_r14-17 (Behringer et al., 1994; Mishina et al., 1996).

1.3.2 Insl3

Descent of the testis from the lower pole of the kidney to the extra-abdominal scrotal sac occurs in two phases, and takes place late on in gestation. Testis descent has therefore been regarded as the completion of sex differentiation (Hughes, 2001). The testis is connected to the abdominal wall by the cranial suspensory ligament (CSL) and caudally, by the gubernaculum. The first phase of testis descent, transabdominal migration, occurs between e_m14.5-18 in mice (Wilhelm and Koopman, 2006), and involves the contraction and thickening of the gubernaculum. This, along with the androgen-dependent regression of the CSL (Emmen et al., 1998), results in the relocation of the testis to the lower abdomen.

Insl3 is a member of the insulin-like hormone superfamily and is produced by the fetal Leydig cells. Insl3 knockout mice were shown to have complete failure of testis descent, an upshot of failed gubernacular development (Adham and Agoulnik, 2004).

The second phase of testis decent, inguino-scrotal descent is normally complete by 20 days after birth in the mouse (Wilhelm and Koopman, 2006). This phase is thought to be androgen dependent, due to the location of testes in patients with hypogonadotrophic hypogonadism and androgen insensitivity syndromes (Bernstein et al., 1988; Lim et al., 2001). Studies

administering the synthetic anti-androgen flutamide, during the window spanning e15.5-18.5, have also demonstrated the importance of testosterone in the completion of inguino-scrotal descent (Kassim et al., 1997; Mylchreest et al., 1999; Shono et al., 1994; Spencer et al., 1991). These data also demonstrate that testosterone is required before, not during this phase of descent (Amann and Veeramachaneni, 2007). Further evidence for testosterone and/or dihydrotestosterone (DHT) playing a role in testis descent, is the extensive expression of AR in the gubernaculum (Staub et al., 2005), the expression of 5α-reductase (George, 1989), and evidence that Insl3 and androgens interact *in vitro* to regulate gubernaculum growth (Emmen et al., 2000).

1.3.3 Testosterone

After the formation of the testis, the production of testosterone is the most important hormonal event of masculinisation. In addition to playing a key role in testis descent, androgens are responsible for the stabilisation and differentiation of the Wolffian ducts (the internal reproductive system) and for regulating the differentiation of external genitalia and the prostate. Androgen action is also essential to masculinise the brain as well as various body tissues and organs.

1.3.3.1 Wolffian duct stabilisation and differentiation

In conjunction with the AMH-induced regression of the Müllerian ducts, masculinisation of the reproductive tract involves the stabilisation and differentiation of the Wolffian ducts. Both of these processes are androgen dependent and once the Wolffian duct has stabilised, testosterone initiates its further differentiation into the epididymis, vas deferens, seminal vesicles and ejaculatory ducts (Welsh et al., 2006). Although testosterone is the principal

androgen involved in Wolffian duct development, it also responds to the weaker androgen, androstenedione (Boehmer et al., 1999).

1.3.3.2 Development of external genitalia and prostate

In mammals, specialised male and female genitalia have evolved to enable internal fertilisation and increase reproductive success. The external genitalia is initially indifferent and is first visible at around e_m10.5 in mice. At around e_m16.5 the development of the external genitalia becomes sex-specific and androgen dependent. It has been reported that DHT, the most potent androgen, is essential for penis formation as 5α-reductase is not only expressed in the genital tubercle mesenchyme, enabling the conversion of testosterone to DHT, but sex reversal syndromes also commonly show a deficiency in 5α-reductase (Wilson et al., 1993). DHT signals through the AR expressed in the developing external genitalia, to induce the elongation of the genital tubercle and fusion of the urethral folds to form the tubular urethra. The scrotum is formed by the caudal movement and fusion of the genital swellings (Wilhelm and Koopman, 2006).

The prostate is a mammalian-specific male accessory sex gland that stores and secretes fluid that contributes to the seminal plasma. The prostate is derived from the indifferent urogenital sinus and its sex-specific development begins at e_m17.5 in response to androgens. Prostate development is similar to that of the genital tubercle (Wilhelm and Koopman, 2006).

1.4 Steroidogenesis

The first steps of steroid synthesis are shared between glucocorticoids, mineralcorticoids and sex steroids (Hiort and Holterhus, 2000). The majority

of steroids are synthesised in the specialised steroidogenic cells of the adrenals, gonads, placenta and brain (Stocco, 2001). However, since male sexual differentiation relies on the production and secretion of testosterone, this section of the review will concentrate on the synthesis of sex steroids, in particular the synthesis of androgens. Where appropriate, the steps of the steroidogenic pathway that are susceptible to interference from the phthalate ester, Di (*n*-butyl) phthalate (DBP), will also be discussed, in preparation for later discussion.

1.4.1 Cholesterol transport

Cholesterol is the precursor of all steroid hormones (Kraemer et al., 2004), providing the backbone of the steroid molecule. The synthesis of testosterone from cholesterol can only occur in the Leydig cells (Payne and Youngblood, 1995), as the adrenal glands are usually only capable of synthesising androstenedione, the testosterone precursor. The first step of steroidogenesis requires the transport of free cholesterol (synthesised *de novo* in the testis), or cholesterol acquired from serum lipoproteins, from the outer to the inner mitochondrial membrane (Thompson et al., 2004). This is where the first enzymatic reaction occurs, catalysed by CYP11a1 (cytochrome P450 side-chain cleavage) enzyme, which is located on the matrix side of the inner mitochondrial membrane (Farkash et al., 1986). Although the outer mitochondrial membrane itself does not provide a barrier to cholesterol, the space between the outer and inner mitochondrial membranes is filled with an aqueous fluid that only permits the passage of water soluble molecules, therefore preventing the passage of lipophilic cholesterol (Stocco, 2001). Consequently, the transport of cholesterol to the inner mitochondrial membrane requires the assistance of scavenger receptor class B type 1 (SRB1)

and steroidogenic acute regulatory protein (StAR), to overcome what is the first and rate-limiting step of steroidogenesis. SRB1 and StAR expression has been shown to be regulated by Sf1 (Cao et al., 1999; Stocco, 2000), and Sf1 has also been shown to regulate the expression of HMG-CoA synthase and HMG-CoA reductase, two enzymes involved in *de novo* synthesis of cholesterol (Mascaro et al., 2000).

1.4.1.1 SRB1

Cholesterol esters are acquired from circulating high-density and low-density lipoproteins (HDL and LDL) and are selectively transported to the inner mitochondrial membrane by SRB1 (Cao et al., 1999; Landschulz et al., 1996). Although cholesterol can be obtained from the conversion of intracellular acetate, this provides an additional and preferable source of cholesterol esters for the fetal testis (Andersen and Dietschy, 1978; Cao et al., 1999). SRB1 gene expression was shown to be significantly reduced in testes from rats exposed to DBP from e_r12-19, and analysed at e_r19 (Barlow et al., 2003).

1.4.1.2 StAR

StAR is rapidly synthesised in response to trophic hormones and acts as an active transporter of cholesterol through the inner mitochondrial membrane (Arakane et al., 1998). Mutations in StAR cause congenital lipoid adrenal hyperplasia, in which adrenal and gonadal synthesis of steroids is impaired at the cholesterol side-chain cleavage step (Bose et al., 1996).

1.4.2 Testosterone biosynthesis

The synthesis of testosterone from cholesterol requires the action of several enzymes, and these fall into two categories; the cytochrome P450 enzymes,

CYP11a1 and CYP17a1; and the hydroxysteroid dehydrogenase (HSD) enzymes, 3β-HSD and 17β-HSD (Payne and Hales, 2004). The P450 enzymes catalyse the hydroxylation and cleavage of the steroid substrate using nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor (Payne and Hales, 2004). The HSD enzymes catalyse the reduction and oxidation of steroid hormones and require $NAD^+/NADP^+$ as electron acceptors (Payne and Hales, 2004). Each P450 enzyme is the product of a single gene, but the HSD enzymes have several isoforms, each a product of a distinct gene (Payne and Hales, 2004).

Once cholesterol has been transported to the inner mitochondrial membrane, the first of several enzymatic reactions take place. The primary steroidogenic enzyme CYP11a1, cleaves a six carbon chain from cholesterol generating the steroid pregnenolone (Thompson et al., 2004). This is the rate-limiting enzymatic step in testosterone biosynthesis (Miller, 1988). *In utero* exposure to DBP results in a significant reduction in the mRNA of CYP11a1, however, it is uncertain whether this is due to a specific DBP effect or a secondary down-regulation caused by decreased cholesterol delivery, resulting from reduced SRB1 and StAR expression (Barlow et al., 2003). Pregnenolone then passes from the mitochondria to the smooth endoplasmic reticulum, where the remaining enzymatic reactions occur (Zirkin and Chen, 2000). A summary of testosterone biosynthesis and the enzymes involved is shown in Figure 1.3. Progression through the testosterone biosynthesis pathway and the enzymes that catalyse these reactions are discussed below.

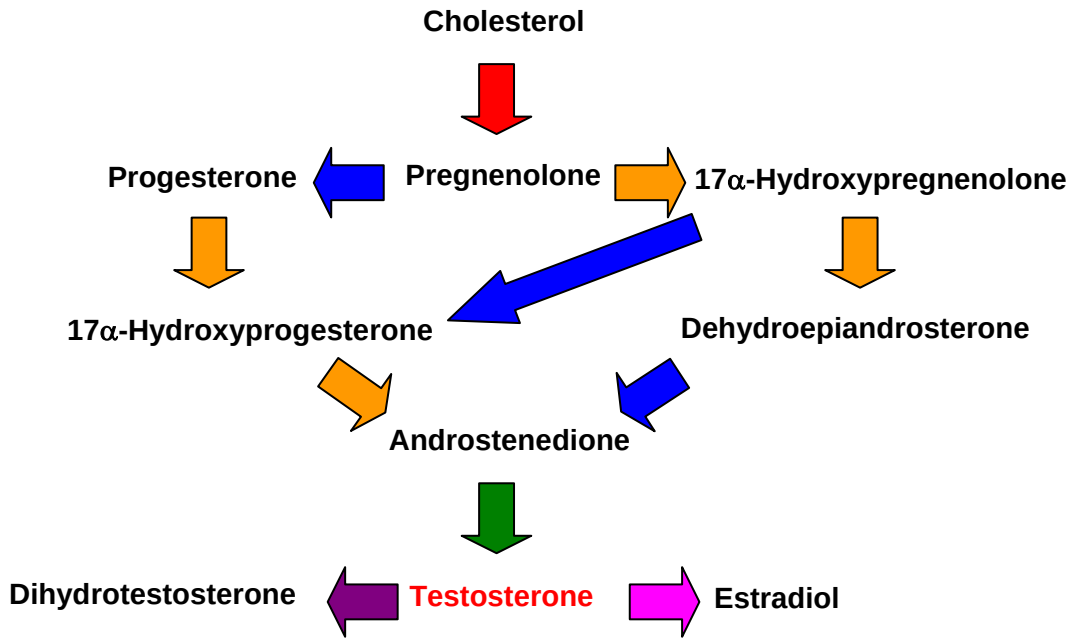

Figure 1.3 Simplified testosterone biosynthesis in a mammalian Leydig cell. The enzymes may catalyse more than one reaction, and the most preferred route of synthesis varies between species. Each enzyme is represented by a different coloured arrow: → CYP11a1, → 3β-HSD, → CYP17a1, → 17β-HSD, → P450 aromatase and → 5α-reductase.

1.4.2.1 Conversion of pregnenolone to androstenedione

The combined enzymatic actions of $\Delta^5$3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD) and CYP17a1 (also known as P450c17 and P450 17α) catalyse the overall conversion of pregnenolone to androstenedione, the precursor of testosterone. This conversion can occur down one of two main pathways; via Δ^5 steroid pregnenolone and its intermediates, 17α-hydroxypregnenolone and dehydroepiandrosterone; or via Δ^4 steroid progesterone and its intermediates 17α-hydroxyprogesterone (Coffey et al., 1972). It is also possible to converge from the Δ^5 pathway to the Δ^4 pathway. In rat testosterone biosynthesis, the Δ^4 pathway, via progesterone, is thought to be the most important pathway (Samuels et al., 1975). All routes of conversion require 3β-HSD and CYP17a1, and because CYP17a1 sequentially

catalyses both 17α-hydroxylase and 17,20lyase activities, it is regarded as the qualitative regulator of steroidogenesis (Hughes, 2001).

Gene expression of both 3β-HSD and CYP17a1 has been shown to be significantly reduced after *in utero* treatment with DBP (Barlow et al., 2003). However, when testes from DBP-exposed rats were incubated with pregnenolone, progesterone or 17α-hydroxyprogesterone, testosterone production increased, although not to the level of the control (Thompson et al., 2004). This suggests that the reduction in supply of intermediate products may lead to the down-regulation of 3β-HSD and CYP17a1, or that even though gene expression was reduced, protein levels were still sufficient to support steroidogenesis (Barlow et al., 2003).

1.4.2.2 17β-HSD

The following enzymes and enzymatic conversions are specific to sex steroid synthesis. The finale step in testosterone synthesis is catalysed by 17β-hydroxysteroid dehydrogenase (17β-HSD, also know as 17-ketosteroid reductase, 17KSR), which reduces androstenedione to testosterone. There are multiple isoforms of 17β-HSD, and type 3 is involved in testis function (Andersson and Moghrabi, 1997). Fetal mice Leydig cells do not express 17β-HSD type 3, and instead it is expressed in the seminiferous tubules. Mouse fetal Leydig cells secrete androstenedione, which is converted to testosterone in the seminiferous tubules (O'Shaughnessy et al., 2000). It is unclear whether this arrangement is peculiar to the mouse or has counterparts in other species.

1.4.2.3 5α-reductase

Once testosterone has been synthesised in the Leydig cells of the testis, it can be further converted to the more potent androgen DHT, by the enzyme 5α-reductase, which is expressed in target tissues (Hiort and Holterhus, 2000). Two isoforms of 5α-reductase have been identified; however, the contribution each isoform makes to male reproductive biology is not yet clear (Henderson and Robaire, 2005).

1.4.3 Regulation of steroidogenesis

Testosterone is synthesised in response to acute and/or chronic stimulation by the pituitary hormone, LH. LH binds to the high-affinity LH receptors on the surface of the Leydig cells, resulting in the activation of adenylate cyclase, followed by an increased production of intracellular cAMP (Payne and Youngblood, 1995). This increase in cAMP leads to the activation of protein kinase A (PKA), which via the phosphorylation of cAMP-dependent transcriptional activators, for example, Sf1, causes an up-regulation of StAR and other steroidogenic genes (Rao et al., 2003). Both acute and chronic effects of LH are mediated via cAMP (Payne and Youngblood, 1995).

As has been previously discussed in section 1.2.7.1, the LH receptor protein is not evident until e_r16.5 in the fetal rat (Majdic et al., 1997) and plasma LH is very low until e_r19.5 in the fetal rat (El-Gehani et al., 1998b), after the onset of testosterone production at e_r15.5 (Warren et al., 1973). In addition to this, *hpg* mice have normal levels of mRNA encoding steroidogenic enzymes, and normal testicular testosterone levels up until birth (O'Shaughnessy et al., 1998), as do LH receptor knockout mice (Zhang et al., 2004). This suggests that fetal testosterone synthesis in the early stages of gestation, at least, is gonadotrophin hormone independent and that Leydig cells are either

constitutively active during early fetal life or stimulated by unknown factors (O'Shaughnessy et al., 1998). Two factors that have been investigated as potential candidates for fetal Leydig cell stimulation are vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP-27). These factors have been shown to stimulate fetal but not adult Leydig cells (El-Gehani et al., 1998a; El-Gehani et al., 1998b). It has also been demonstrated that adrenocorticotropic hormone (ACTH) stimulates androgen production, by fetal-type Leydig cells, in the fetal/neonatal mouse testis, through the melanocortin type 2 receptor (MC2R) (Johnston et al., 2007).

1.4.3.1 HPG-axis

The hypothalamic-pituitary-gonadal (HPG) axis contains three component parts; the gonadotrophin releasing hormone (GnRH) neurons that project from the hypothalamus of the brain, the gonadotroph cells of the anterior pituitary and the somatic cells (Sertoli cells and Leydig cells) of the gonads (Figure 1.4) (IPCS, 2002). The HPG axis is vital to the development and regulation of the mature reproductive system. It regulates the synthesis of follicle stimulating hormone (FSH), which plays a role in neonatal Sertoli cell proliferation and spermatogenesis, and the release of luteinising hormone (LH), which in the adult, at least, regulates testosterone synthesis.

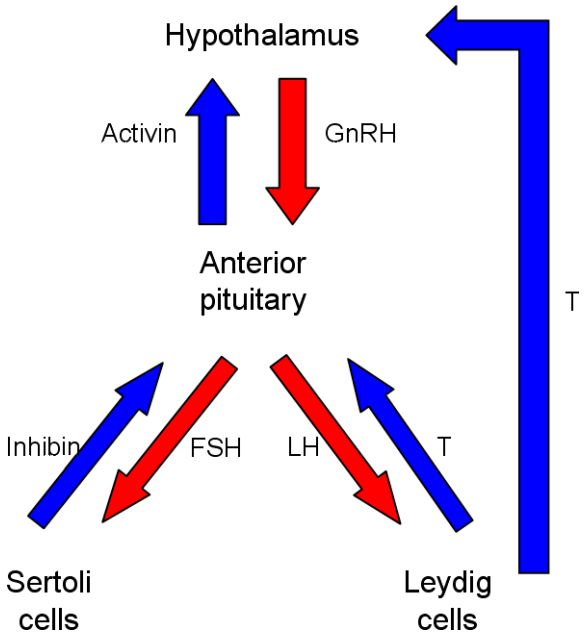

Figure 1.4 Summary of the HPG axis in the male. The hormones represented by red arrows are stimulatory, and those represented by blue arrows are inhibitory, once their circulating concentrations reach a critical level.

The hypothalamus is the integrative centre of the hormone axis and due to its pulsatile secretion of the peptide hormone GnRH (Kimura and Funabashi, 1998), from the terminals of the GnRH neurons, it is considered the pulse generator for the rhythmic secretion of pituitary and gonadal hormones. GnRH then travels via the portal vascular system, to the anterior pituitary, where it stimulates the secretion of the gonadotrophic hormones; LH and FSH, from the gonadotroph cells. Recent evidence suggests that the HPG axis is further regulated by kisspeptin-54, a peptide product of the KiSS-1 gene that acts through the G protein-coupled receptor GPR54. Kisspeptin potently stimulates the HPG axis, via the hypothalamus, and in adult rats this results in the increase in circulating levels of LH and testosterone (Patterson et al., 2006).

1.4.3.1.1 Gonadotrophic hormones

LH and FSH are peptide hormones, and although they are named after their roles in female reproduction, they are equally important in the male. GnRH initiates the secretion of LH and FSH by binding to high-affinity receptors on the gonadotroph cells of the anterior pituitary. The effects of GnRH on LH and FSH secretion are very different; LH release occurs rapidly in response to acute GnRH pulses, whereas FSH release is sluggish, and may take several hours to peak (Bousfield et al., 1994; Crowley, 1999). Regulation of LH and FSH secretion involves a complex interaction of GnRH stimulation and negative feedback regulation by the gonadal sex steroids (e.g. testosterone), stimulated by LH, and inhibin-B from the Sertoli cells, stimulated by FSH. The sex steroids negatively regulate LH via effects on the hypothalamus and anterior pituitary, and also exert some negative feedback on FSH secretion. In contrast, Inhibin-B selectively inhibits FSH secretion via effects on the anterior pituitary (Crowley, 1999). Pituitary factors, for example, activin, probably also play a role in negative feedback (Figure 1.4; (Blumenfeld and Ritter, 2001). Each target cell in the HPG axis also regulates its own responsiveness. If stimulation becomes abnormally frequent, the cells become less sensitive accordingly (IPCS, 2002) a process referred to as desensitization.

LH and FSH are members of the glycoprotein family, along with thyroid stimulating hormone (TSH) and chorionic gonadotrophin (hCG). They exert their effects on their target cells in the testis by binding to their respective receptor, activating adenylate cyclase and consequently, increasing intracellular cAMP.

1.5 Testicular dysgenesis syndrome

Disorders of human male reproductive health which manifest at birth (cryptorchidism and hypospadias) or in young adulthood (testicular germ cell cancer and low sperm counts) are common and may be increasing in incidence in the Western world (Sharpe and Skakkebaek, 2003). Although testicular germ cell cancer and low sperm counts do not manifest until adulthood, it is generally accepted that testis germ cell cancers arise from abnormal fetal germ cells (Rajpert-De Meyts et al., 1998; Rorth et al., 2000) and it has also been suggested that low sperm counts occur via a reduction in Sertoli cell proliferation in fetal life, which leads to a reduction in the number of germ cells that can be supported through spermatogenesis (Sharpe, 2006). Each of the above mentioned disorders are risk factors for each other and they share other risk factors such as intrauterine growth restriction, and impaired androgen production/action in fetal life (Sharpe and Skakkebaek, 2003). Based on this evidence, it has been hypothesised that these disorders comprise a testicular dysgenesis syndrome (TDS) in which abnormal testis development (dysgenesis) leads secondarily to hormonal or other malfunctions of the Leydig and/or Sertoli cells during male sexual differentiation, leading in turn to increased risk of the TDS disorders (Sharpe and Skakkebaek, 2003; Skakkebaek et al., 2001) (Figure 1.5). The order in which the disorders of TDS are presented in Figure 1.5, depict their frequency, the most common being low sperm counts and cryptorchidism and the rarest, being testis germ cell cancer.

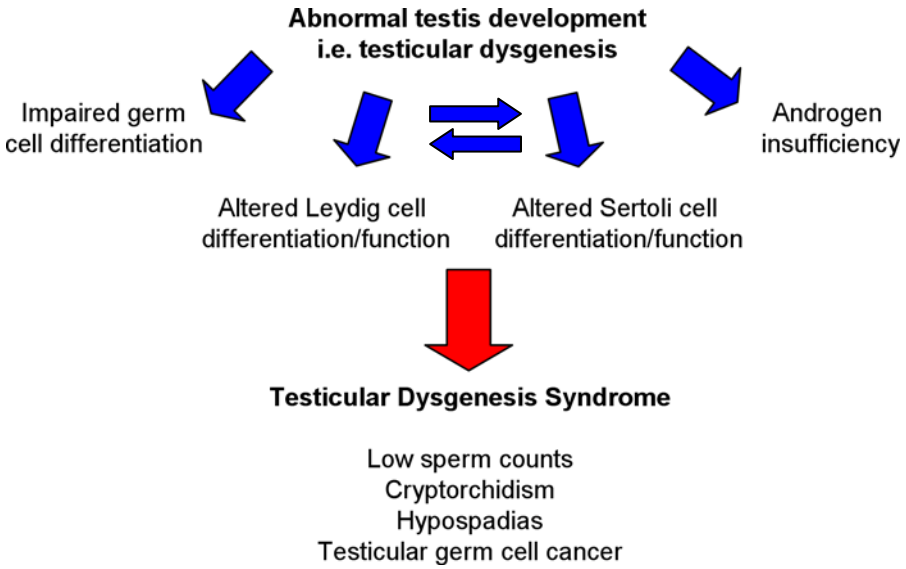

Figure 1.5 Schematic representation of the potential links between testis development and the clinical manifestations of TDS. Adapted from (Fisher, 2004).

1.5.1 Animal model for TDS

The mechanisms that give rise to testicular dysgenesis syndrome are unknown and are impossible to study in the human fetus. However, a comparable animal model has been identified, enabling investigation into the mechanistic pathways behind TDS.

1.5.1.1 Phthalates

Phthalates are ubiquitous chemicals used in the production of PVC products and in personal care products such as nail varnish (Foster et al., 2001). *In utero* exposure of rats to certain phthalates, such as Di (n-butyl) phthalate (DBP) and Di (2-ethylexyl) phthalate (DEHP), results in a range of reproductive abnormalities comparable to human TDS (Barlow and Foster, 2003; Ema et al., 1998; Ema et al., 2000; Fisher et al., 2003; Mylchreest et al., 1998; Mylchreest et al., 2000; Parks et al., 2000). This review will concentrate on the effects of *in utero* exposure to DBP, as this was the phthalate used in studies in this thesis.

1.5.1.2 DBP

In utero exposure to DBP has been shown to induce cryptorchidism, hypospadias, impaired spermatogenesis and reduced fertility in male rats postnatally (Barlow and Foster, 2003; Ema et al., 1998; Ema et al., 2000; Fisher et al., 2003; Mylchreest et al., 1998; Mylchreest et al., 1999). This spectrum of disorders closely parallels those disorders seen in human TDS, with the exception of testicular germ cell cancer, and as such provides a useful model for the study of TDS, and raises the possibility that phthalate exposure could contribute to TDS disorders in the human. The postnatal DBP-induced alterations are preceded by several changes to the fetal testis. *In utero* exposure to DBP causes a marked reduction in Leydig cell hormone (testosterone and Insl3) production, widespread occurrence of multinucleated fetal germ cells (Fisher et al., 2003; McKinnell et al., 2005; Parks et al., 2000; Shultz et al., 2001; Wilson et al., 2004), abnormal migration/aggregation of fetal Leydig cells which is thought to result in postnatal focal dysgenesis of seminiferous cords/tubules (Fisher et al., 2003; Mahood et al., 2005; Mahood et al., 2006) and the presence of apparently immature Sertoli cells and Sertoli cell only (SCO) tubules (Fisher et al., 2003).

The abnormal focal dysgenetic areas observed in the testis after DBP exposure have also been reported in biopsies of the contralateral testes of testicular germ cell cancer patients. These contralateral testes frequently exhibit undifferentiated Sertoli cells, Leydig cell hyperplasia, SCO tubules, abnormal seminiferous cord morphology and carcinoma in situ (CIS) cells, the pre-invasive precursor cells of testicular germ cell tumours (Hoei-Hansen et al., 2003; Holm et al., 2003; Skakkebaek et al., 2003). It is possible that the abnormal multinucleated gonocytes observed after DBP exposure, may be linked in some way to the abnormal CIS cells seen in human testes, which are

thought to originate in fetal life from primordial germ cells or early gonocytes (Sonne et al., 2004).

The reduction in testicular testosterone levels observed after DBP exposure is also in keeping with the impaired androgen production/action which is thought to play a key role in human TDS (Sharpe and Skakkebaek, 2003). *In utero* exposure to DBP also results in a reduction in anogenital distance (AGD), indicative of reduced or impaired androgen action (Mylchreest et al., 1998). It is accepted that hypospadias and cryptorchidism result from a reduction in the Leydig cell hormones, testosterone and Insl3 respectively, however, recent evidence suggests that reduced fetal androgen production/action could also play a role in low sperm counts in adulthood. The Sertoli cells can only support a finite number of germ cells through spermatogenesis, so Sertoli cell number per testis determines how many germ cells can be supported, and consequently, the spermatogenic capability of the testis (Sharpe et al., 2003). Sertoli cell number is determined during perinatal life and, as discussed previously, testicular feminised (*tfm*) mice were reported to have significantly reduced Sertoli cell numbers at birth (Johnston et al., 2004). This suggests that androgens play a role in Sertoli cell proliferation and the determination of the spermatogenic capabilities of an individual, and that if androgen production/action was suppressed, as is postulated to be the case in human TDS and actually occurs in the DBP animal model of TDS, this may result in reduced sperm counts.

Although it is not certain that data from the DBP animal model of TDS will provide accurate insights into human TDS, it is a rational starting point from which to identify potential pathways and mechanisms that are likely to be important and could then possibly be studied in the human.

1.5.2 Maternal smoking and reduced sperm counts

In humans, maternal smoking has been shown to cause a reduction of testis size and sperm count in the mature male offspring. It has been reported that smoking ten or more cigarettes a day during pregnancy, led to a 30-48% reduction in sperm counts and testis size in adulthood in exposed males (Storgaard et al., 2003), and blood concentrations of Inhibin B, said to be primarily determined by Sertoli cell number (Ramaswamy et al., 1999), were decreased by 24%, suggesting that these reductions could be the result of reduced Sertoli cell number. Another study of over 1,700 men, reported that those men exposed to cigarette smoke *in utero*, exhibited a 20.1% reduction in sperm concentrations, a 24.5% reduction in total sperm count and a 1.15 ml smaller testis size (Jensen et al., 2004). These findings suggest that *in utero* exposure to cigarette smoke causes a reduction in testis size and sperm counts by disrupting perinatal Sertoli cell proliferation. Recent evidence suggests this might involve alterations of the desert hedgehog (Dhh) signalling pathway (Fowler et al., 2007).

2.1.1.1 PAHs

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxic organic chemicals that are produced by the incomplete combustion of hydrocarbons, including fossil fuels (Vinggaard et al., 2000). PAHs are also present in tobacco smoke (Miller et al., 2004). The adverse effects of PAHs are mediated via the aryl hydrocarbon receptor (AhR) (Gonzalez and Fernandez-Salguero, 1998), which has been reported to be stage-specifically immunoexpressed in Sertoli cells and Leydig cells of the rat testis (Schultz et al., 2003). In addition to this, a number of PAHs have been shown to act as anti-androgens *in vitro* in transfected cells (Vinggaard et al., 2000), and activation of the AhR is

thought to antagonize androgen action via interaction with the AR (Kizu et al., 2003). This suggests that PAHs in tobacco smoke may antagonise androgen action in the fetal testis and disrupt Sertoli cell proliferation, leading to a reduction in Sertoli cell number and a concurrent reduction in testis weight and sperm count.

1.5.2.1 DMBA

One of the PAHs shown to have anti-androgenic properties *in vitro*, is 7,12-dimethylbenz[a]anthracene (DMBA). DMBA is also one of the main components of tobacco smoke (Hoffmann and Hecht, 1990). Consequently, DMBA was chosen as a candidate chemical to investigate the role of maternal smoking in reduced Sertoli cell number and sperm counts in this thesis. It should be noted however, that there are many other PAHs present in tobacco smoke, such as benzo[a]pyrene, and DMBA is just one possible candidate.

1.5.3 IUGR

Maternal smoking not only causes a reduction in the testis weight and sperm counts of mature male offspring, it also results in the reduced birth weight of offspring (Jensen et al., 2004), and reduced birth weight was also reported to contribute to testis size and semen quality, although not to the same extent as maternal smoking (Jensen et al., 2004). As was mentioned earlier in section 1.5, intrauterine growth restriction (IUGR) is also one of the risk factors associated with all four disorders of TDS, including low sperm counts (Sharpe and Skakkebaek, 2003; Skakkebaek et al., 2001).

1.5.3.1 Dexamethasone

Glucocorticoid treatment during pregnancy has been shown to reduce birth weight in animals and humans (Newnham et al., 1999; Reinisch et al., 1978).

The human fetus is protected from the active glucocorticoid, cortisol (corticosterone in the rat), by 11β-HSD2, a placental enzyme that converts cortisol into the inactive cortisone (Drake et al., 2005). Human fetuses with IUGR have increased levels of cortisol, indicating that endogenous cortisol may play a role in IUGR (Goland et al., 1993).

Dexamethasone is a synthetic glucocorticoid that acts as a poor substrate for 11β-HSD2, and consequently has been previously shown to cause IUGR in rats, when administered during pregnancy (Drake et al., 2005). It has also been reported that prenatal exposure to dexamethasone causes a reduction in AGD (Holson et al., 1995), indicative of reduced testosterone levels/action. This was confirmed after postnatal exposure resulted in a reduction in testosterone levels in immature (postnatal day 35) rats (Page et al., 2001). Consequently, *in utero* exposure to dexamethasone provides an ideal animal model for further investigation into the role of IUGR and reduced testosterone levels in TDS.

1.6 Role of androgens in testis development

The initial formation of the testis, as has been discussed in this review, is hormone independent. This has consequently led to the opinion that fetal testis development is also hormone independent. However, there is increasing evidence to suggest that hormone action may play a role in fetal testis development.

In addition to evidence that hormonal (endocrine) disruption plays a role in the fetal origins of TDS (Sharpe and Skakkebaek, 2003; Skakkebaek et al., 2001), patients with complete androgen insensitivity syndrome (CAIS; also known as 'testicular feminisation' or *tfm*), due to an inactivating mutation in the AR, may exhibit focal areas of testicular dysgenesis (Hannema et al.,

2006). CAIS patients are also at a higher risk of developing testicular germ cell cancer (Cools et al., 2005; Verp and Simpson, 1987), and are cryptorchid. This implies that reduced androgen production/action may be in involved in the formation of dysgenetic areas within the testis.

As discussed earlier, *tfm* mice show a 50% reduction in Sertoli cell number at birth (Johnston et al., 2004), suggesting that androgens are involved in Sertoli cell proliferation. However, fetal Sertoli cells do not express AR (Williams et al., 2001), so it is therefore hypothesised that androgens exert their proliferative effect on Sertoli cells via the AR positive fetal peritubular myoid cells (Sharpe, 2005; Tan et al., 2005).

1.6.1 Androgen receptor

The androgen receptor is a hormone-activated DNA-binding transcription factor that regulates androgen targeted genes (Hiort and Holterhus, 2000). The AR, like other members of the steroid receptor superfamily, is divided into three main functional domains that are involved in transcriptional regulation, DNA and ligand binding. The large N-terminal domain is the least conserved and contains an activation function (AF1) region, which is independently involved in gene transactivation (Hughes, 2001). The N-terminal precedes the DNA binding domain, which is followed by the C-terminus, which also contains an activation function (AF2) region, as well as mediating heat shock protein interactions, dimerisation, nuclear localisation signalling and ligand binding (Hughes, 2001).

Prior to ligand binding, the AR is an inactive oligomer complexed to heat shock proteins (e.g. Hsp 90 and Hsp 70) and is located in the cytoplasm of the target cell (Jenster et al., 1993; Kimura et al., 1993). Testosterone passively diffuses into the cytoplasm of the target cell and can be converted by 5α-

reductase, into DHT, which also acts via the AR. Upon ligand binding, the oligomeric complex dissociates from the heat shock proteins, the receptor undergoes further phosphorylation and moves to the nucleus where homodimerisation occurs (Hiort and Holterhus, 2000). The homodimerisation of the AR is an androgen-dependent process that is mediated by distinct sequences within the second zinc finger of the DNA binding domain and specific N-C terminal interactions (Beato et al., 1996). The AR homodimer then binds to the androgen responsive elements, palindromic sequences within the promoter regions of androgen regulated target genes, where, under the influence of transcription factors and with the recruitment of RNA polymerase II, target gene transcription is initiated or repressed and results in distinct biological effects (Hiort and Holterhus, 2000) (Figure 1.6).

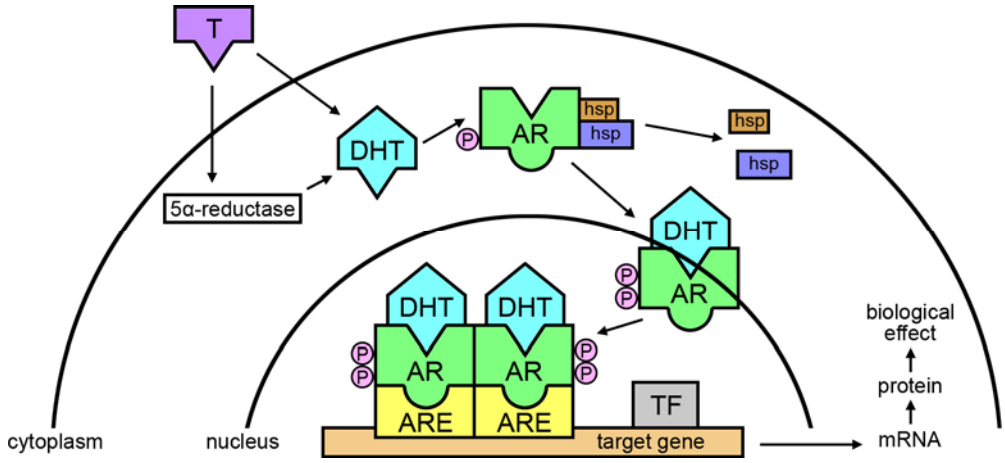

Figure 1.6 A summary of the events involved in androgen action. In the absence of a ligand, the AR is located in the cytoplasm and bound to heat shock proteins (hsp). Testosterone (T) passively diffuses into the cytoplasm and either binds to the AR or is converted to DHT by 5α-reductase. Upon ligand binding the AR undergoes further phosphorylation, disassociates from the hsps, enters the nucleus and becomes a homodimer. The AR obtains its specific response by binding to androgen responsive elements (ARE) and under the influence of transcription factors (TF) and with recruited RNA polymerase II (not shown), initiates the up- or down-regulation of target genes. Adapted from (Hiort and Holterhus, 2000).

The AF regions also play a role by interacting with co-regulators, a group of intermediary proteins. Protein: protein interactions are formed in a ligand-dependent manner, and can increase (co-activator) or decrease (co-repressor) gene transcription (McKenna et al., 1999; Robyr et al., 2000).

Androgens have been shown to enhance the expression of AR in the developing reproductive tract by increasing the density and frequency of AR positive cells (Bentvelsen et al., 1995).

In addition to the transcriptional or genomic mode of androgen action, recent evidence suggests that androgens, like oestrogen and progesterone, can exert rapid non-genomic effects. Testosterone can induce second messenger signal transduction cascades within seconds, which is too fast to involve changes in transcription or protein synthesis (Heinlein and Chang, 2002). Evidence from the postnatal rat has demonstrated that the addition of testosterone to

cultured Sertoli cells stimulates the MAP kinase signalling pathway and causes the phosphorylation of the cAMP response element binding protein, a modification that is required for Sertoli cells to support spermatogenesis (Fix et al., 2004). Intracellular calcium levels are also increased within seconds of Sertoli cell androgen stimulation (Fix et al., 2004). Although there is currently no evidence to suggest that androgens have non-genomic effects in fetal life, as this has not yet been investigated, it is possible that non-genomic androgen effects could play a role in fetal development.

1.6.2 Impaired androgen action

As demonstrated by this review, androgen action is vital for the masculinisation of the male embryo, specifically the internal reproductive system and external genitalia, and may also play an important role in normal testis development. In addition to the treatments already discussed; DBP, DMBA and dexamethasone, which have either been shown to reduce testicular testosterone levels, or are reported to antagonise androgen action, this final section of the review will concentrate on the disruption of normal androgen action via a variety of mechanisms.

1.6.2.1 Androgen receptor mutation

Androgen insensitivity (AIS) is an X-linked genetic disease, and is defined by either the complete (CAIS) or partial (PAIS) absence of androgen responsiveness in XY males with otherwise normal testis determination and androgen biosynthesis (Ahmed et al., 2000; Quigley et al., 1995). CAIS patients have testes and normal testosterone production but show female phenotypic characteristics. PAIS produces a spectrum of abnormalities in male development. Over 300 AR mutations have been identified in the AR of AIS patients (Hiort and Holterhus, 2000), including large deletions, point

mutations and aberrant splicing (He et al., 1991). A large proportion of these mutations affect the AR ligand binding domain (Hughes, 2001).

Testicular feminised (*tfm*) mutant mice are used as an animal model for human CAIS. It has been reported that the absence of androgen responsiveness seen in *tfm* mice, is a result of a frame-shift mutation in the coding region of the AR gene, which disrupts the normal reading frame of AR translation (He et al., 1991).

1.6.2.2 Defects in androgen production

Androgen biosynthesis can be disrupted via a number of possible mutations in the genes that encode the steroidogenic enzymes and results in a range of phenotypes that can vary from complete XY sex reversal, to varying degrees of undermasculinisation (Hughes et al., 2006). One example involves mutations in the steroidogenic enzyme 17β-HSD type 3, which converts androstenedione to testosterone. So far 17 mutations in this gene have been reported, all of which give rise to severely undermasculinised males (Hughes et al., 2006).

1.6.2.3 Anti-androgenic compounds

Androgen action can also be disrupted by the administration of anti-androgenic compounds that prevent or inhibit androgen action, by competitively binding to the AR, preventing androgens from binding and thus blocking androgen mediated AR activation. There are a number of anti-androgens, some environmental, for example the pesticides linuron and vinclozolin, and others that are used as drugs, for example, flutamide, which has been used therapeutically to treat androgen-dependent prostate cancer

(Murphy et al., 1991). This review will concentrate on flutamide, as this was the anti-androgen used in studies in this thesis.

1.6.2.3.1 Flutamide

Flutamide (4'-nitro-3'-trifluoromethyl-isobutyranilide) is a synthetic nonsteroidal AR antagonist that competitively blocks the action of both testosterone and dihydrotestosterone (DHT) (Peets et al., 1974). Flutamide has not only been used therapeutically; it has also been administered to rats as a tool for studying hormone-dependent male reproductive development (McIntyre et al., 2001). *In utero* exposure to flutamide has been shown to result in reduced reproductive organ weights, to induce major reproductive tract abnormalities in the male including the complete prevention of masculinisation of external genitalia, including hypospadias and AGD (Imperato-McGinley et al., 1992; McIntyre et al., 2001), and to cause cryptorchidism and nipple retention (Imperato-McGinley et al., 1992). Although abnormal testicular morphology, including testicular hypoplasia and seminiferous tubule degeneration, has been reported in the adult rat after *in utero* exposure to flutamide (Foster and Harris, 2005; McIntyre et al., 2001), the effects on fetal testis development have, as of yet, not been reported.

1.6.2.4 Exogenous androgen exposure

In addition to treatments that block androgen action and interfere with androgen production, the exogenous androgen, testosterone propionate (TP), was administered *in utero*, in studies in this thesis, to investigate whether the effects of suppressed testicular testosterone levels could be attenuated by enhancing the levels of testicular testosterone. It has been reported that *in utero* exposure to TP results in the masculinisation of what should be female

genitalia, including increased AGD (Swanson and Werff ten Bosch, 1965), and IUGR (Fritz et al., 1984; Slob et al., 1983).

1.7 Aims of thesis

TDS arises when maldevelopment (dysgenesis) of the fetal testis results in hormonal malfunctions and abnormal development and function of the somatic cells. Evidence from *tfm* mice suggests that the suppressed intratesticular testosterone levels postulated to occur in TDS might account for subsequent low sperm counts, via a reduction in perinatal Sertoli cell proliferation/number. Sertoli cells do not express androgen receptors in fetal life, so it is hypothesised that any androgen effects on Sertoli cell number occur indirectly, via the AR positive peritubular myoid cells. Evidence from the DBP-treated rat model for TDS suggests that reduced androgen action may also play a role in testicular dysgenesis, as in patients with CAIS, in whom focal areas of testicular dysgenesis have been reported.

The aim of the experimental work detailed in this thesis was to establish if reduced androgen levels/action in the fetal rat testis contribute to putative testicular dysgenetic features, namely reduced Sertoli cell number (chapter 4), occurrence of multinucleated gonocytes or abnormal aggregation of fetal Leydig cells, the precursor of focal dysgenesis (chapter 5). Pregnant rats were exposed to treatments or co-treatments expected to manipulate intratesticular testosterone levels (DBP, TP) or action (flutamide, DMBA) or to induce intrauterine growth restriction (dexamethasone; chapter 6), another risk factor for TDS. The same endpoints were evaluated in mice with inactivation of the androgen receptor (*tfm* or ARKO mice). As androgen action is assumed to be mediated indirectly, via the peritubular myoid cells, changes in peritubular myoid cell number and function were investigated in

testes with suppressed androgens (chapter 7). *In vitro* studies were also used to investigate the role of androgens in Sertoli cell proliferation (chapter 4). In addition, potential non-androgen related mechanisms of DBP action were investigated using Taqman RT-PCR to determine the mRNA expression of key developmental genes after exposure to DBP (chapter 7).

2 General Materials and Methods

2.1 Animal work

All animal work was carried out in accordance with the UK Home Office Animal Experimentation (Scientific Procedures) Act 1986 under project licence 60-3259. The animals used were Wistar rats, purchased from Harlan UK and then bred within our own animal facility to generate stock. Mark Fisken provided the day-to-day animal husbandry and also performed the majority of licensed procedures, although I was able to perform some procedures under my personal licence 60-9997.

2.1.1 Welfare conditions

Animals were housed in a room where light was provided for 12 hours per day, from 7am-7pm; the temperature was kept between 20-25°C and the average humidity was 55%. Fresh tap water and food (Soy-free, SDS, Dundee, Scotland) were available *ad libitum*. The rats were kept in clear sided, solid bottom cages, with females typically housed in groups of six and stud males housed individually. When mating was required, a male and female were placed temporarily in a cage with a mesh grid bottom that allowed the copulatory plug to fall to the tray below.

2.1.2 Timed-mating

Timed-mating was used to ascertain the date of conception and gestational stage of pregnant rats. One male and one female were placed together in a grid bottomed cage at the end of the working day (usually about 4pm), and the tray beneath the cage examined for copulatory plugs the following morning, as mating usually takes place at night. Once a copulatory plug was detected, mating was assumed to have occurred. The date was recorded and

designated as embryonic day (e) 0.5; the male was then separated from the female. Female rats have a four to five day oestrus cycle, so copulation could take up to five days to occur.

Rats become sexually mature at ~6 weeks of age, but all rats mated for use in this thesis were a minimum of 10 weeks old, usually three months old. Wherever possible, proven fertile males and females were mated to reduce the risk of non-pregnancy.

2.2 *In vivo* treatments

The chemicals administered to pregnant female rats for studies *in vivo* were Di (*n*-butyl) phthalate (DBP), flutamide, 7,12-diemethyl benz[a]anthracene (DMBA), testosterone propionate (TP) and dexamethasone, each given alongside their respective control vehicles.

DBP, flutamide and DMBA were administered daily by oral gavage using a 10-12cm long 15-16G blunt ended steel gavage cannula (Medicut, Sherwood Medical Industries Ltd., UK), attached to a disposable plastic 1 ml syringe (B-D Plastipak). TP and dexamethasone were administered daily by subcutaneous injection using a 25G, 0.5 x 16mm needle (BD Microlance) attached to a plastic 1 ml syringe. Pregnant dams were weighed prior to dosing so the correct volume of chemical for bodyweight could be calculated. Treatments were administered between 0900 and 1030 hrs and treated animals were checked at regular intervals for signs of discomfort or toxicity.

2.2.1 Di (*n*-butyl) phthalate (DBP)

The principal dose of DBP (Sigma) used this thesis was 500mg/kg bodyweight, as this dose has previously been shown to induce a high incidence of TDS-like disorders, including Leydig cell aggregation in fetal

testes, dysgenetic areas in adulthood and the occurrence of multinucleated gonocytes (Fisher et al., 2003; Mahood et al., 2005; Mahood et al., 2006; Mylchreest et al., 1998). For dose response studies, doses of 100, 20 and 4mg/kg were also administered. Most typically, treatment commenced at e13.5, around the time of seminiferous cord formation, but other treatment windows were also investigated. These consisted of exposure from e13.5 to e15.5 (early window), from e15.5 to e17.5 (middle window) from e19.5 to e20.5 (late window) and e11.5 to e20.5 (extended window). All pups treated in these regimes were killed at e21.5.

DBP was diluted to the required dose with corn oil (supermarket bought), in line with previous *in vivo* DBP studies from this and other laboratories. It was assumed that DBP and corn oil had comparable densities (1g/ml) so when making up a dose of 500mg/kg bodyweight, for example, 5ml DBP was made up to 10ml with corn oil and given at 1ml/kg bodyweight. Treatments were freshly made for each study, kept at room temperature in an airtight container, and appropriately disposed of at the end of the study.

For all DBP treatment age ranges, control animals received 1ml/kg bodyweight corn oil. All dosing regimes are summarised below.

Table 2.1 Summary of the DBP treatment and sampling used for the present studies.

Dose (ml DBP/kg bodyweight)	Treatment window	Kill age
500	e13.5-20.5	e15.5, 17.5, 19.5 or 21.5
100	e13.5-20.5	e21.5
20	e13.5-20.5	e21.5
4	e13.5-20.5	e21.5
500	e13.5-15.5	e21.5
500	e15.5-17.5	e21.5
500	e19.5-20.5	e21.5
500	e11.5-20.5	e21.5

2.2.2 Flutamide

Flutamide is a synthetic anti-androgenic agent that competitively blocks the action of both testosterone and dihydrotestosterone (DHT) at the level of the androgen receptor (Peets et al., 1974). The dose of flutamide administered for studies in this thesis was 100mg/kg. In preliminary studies, a dose of 50mg/kg was used, based on previous evidence that administration of >24mg/kg to pregnant rats induced major reproductive tract abnormalities in the male including the complete prevention of masculinisation of external genitalia and anogenital distance (AGD; (Imperato-McGinley et al., 1992; McIntyre et al., 2001). However, since the 50mg/kg dose failed to have an effect on Sertoli cell number (see chapter 4), the higher dose of 100mg/kg was used instead.

Flutamide treatment began at e15.5, the age at which testosterone production commences (Warren et al., 1973). Flutamide is insoluble in oil, so was dissolved in 2.5% dimethyl sulphoxide (DMSO; Sigma), diluted in corn oil, then administered via oral gavage at 1ml/kg bodyweight. Control animals received the DMSO/corn oil vehicle by gavage. Flutamide was freshly prepared for each study, kept at room temperature between doses and disposed of appropriately at the end of each study.

2.2.3 7,12-diemethylbenz[a]anthracene (DMBA)

DMBA is a polycyclic aromatic hydrocarbon (PAH) found in cigarette smoke. The dose administered for studies in this thesis was 400μg/kg. An initial dose of 200μg/kg was used in preliminary studies, but as it showed no effect on Sertoli cell number (see chapter 4), it was increased to 400μg/kg. Dose levels were chosen based on previous studies which demonstrated that a single dose of 1-20mg/kg was sufficient to induce DNA damage and mammary

tumours in adult rats (Manjanatha et al., 1996; Zaccheo and Di Salle, 1989). A lower dose was selected to avoid inducing overt DNA damage but to reflect chronic lower level exposure that would typify PAH exposure from smoking. There is *in vitro* evidence that DMBA may have anti-androgenic properties (Kizu et al., 2003; Vinggaard et al., 2000), therefore exposure was started at e15.5, when steroidogenesis begins.

DMBA was freshly made up for each study, and because DMBA is soluble in oil, it was diluted in corn oil (400μg per ml corn oil). For complete solubilisation the mixture was gently warmed in a glass vial on a hot plate. DMBA was administered via oral gavage at 1ml/kg bodyweight; it was kept at room temperature between doses and disposed of appropriately at the end of each study.

2.2.4 Testosterone proprionate (TP)

TP was administered to pregnant rats from e14.5 to e20.5 in accordance with previous studies (Wolf et al., 2004), in order to manipulate intratesticular testosterone levels. The initial dose used, 5mg/kg, was selected based on studies by (Wolf et al., 2002), who showed that administering 1mg of TP per pregnant rat from gestational day 14 to 19 increased fetal testosterone by 80% and increased female anogenital distance (AGD), without a high incidence of toxicological effects. However, as this preliminary dose induced no change in Sertoli cell number (Chapter 4), a higher dose of 20mg/kg was administered for the rest of the studies.

TP is insoluble in water so was dissolved in corn oil (20mg TP per ml corn oil) at the start of each study. In order to keep the TP in solution between doses, it was stored on a Cozee Comfort heating mat (Burco Dean Appliances Ltd, Burnley, UK) setting 1 at 29°C. TP was administered at

1ml/kg bodyweight by subcutaneous injection using a disposable plastic 1ml syringe fitted with a 25G needle. Any excess TP was disposed of appropriately at the end of each study.

2.2.5 Dexamethasone

Dexamethasone is a synthetic glucocorticoid that has been previously shown to cause intrauterine growth restriction (IUGR), when administered during fetal life (Drake et al., 2005). IUGR is one of the risk factors for TDS. The dose of dexamethasone used in these studies was 100μg/kg (in accordance with (Drake et al., 2005), and was made up in 0.9% saline containing 4% ethanol. Consequently, to make 50ml, for example, 10mg dexamethasone was dissolved in 2ml ethanol and then added to 48ml saline. Dexamethasone was administered daily by subcutaneous injection, and was given at 0.5ml/kg bodyweight, using a syringe fitted with a 25G needle. Dexamethasone treatment was administered daily, from e13.5 to e21.5, to allow direct comparison with DBP studies.

2.2.6 5-Bromo-2'deoxyuridine-5'-monophosphate (BrdU)

Cell division occurs in four stages, G_1, S, G_2 and mitosis, and it is during the S (synthetic) phase that DNA replication takes place. BrdU is a synthetic analogue of thymidine, a nucleotide specific to DNA. If BrdU is present, it substitutes thymidine and becomes incorporated in the newly synthesised DNA. Inclusion of BrdU can be identified using BrdU specific antibodies and thus proliferating cells can be recognized. Counting labelled and unlabelled nuclei allows the calculation of a Proliferation Index (PI), whereby the number of proliferating cells of a certain type is expressed as a percentage of the total number of that cell type within the sample analysed (section 2.7.3).

BrdU was administered at a dose of 100mg/kg bodyweight in 2ml/kg saline (0.9% NaCl, w/v) via intraperitoneal injection. Solubilisation was achieved most efficiently if the saline was first heated to boiling point in the microwave. Once the BrdU was added, the solution was allowed to cool to body temperature before being injected into the animals. Injection took place 1.5 hrs prior to kill.

2.3 Androgen receptor knockout (ARKO) mice and testicular feminised (*tfm*) mice

The ARKOs were generated in Belgium by Karel De Gendt, using Cre/*loxP* technology (De Gendt et al., 2004). Cre/*loxP* technology relies on the ability of the P1 bacteriophage cyclization recombination (Cre) recombinase gene to affect recombination between pairs of *loxP* sites. Cre/*loxP* mice are most typically created by breeding a Cre mouse with a *loxP* mouse. The Cre mouse contains a Cre-recombinase transgene under the control of a tissue specific promoter. In the case of the ARKO mice, these were male mice (C57BL/6) expressing Cre-recombinase ubiquitously under the control of the phosphoglycerate kinase-1 (PGK) promoter.

The *loxP* mice contain two *loxP* sites that flank the genomic segment of interest, hence it is said to be 'floxed'. To generate the ARKOs, female mice (129/Swiss), heterozygous for the floxed AR allele (AR^{flox}/AR^{+}) were used. Because it is impractical to flank the entire gene with *loxP*, a vital exon is usually selected for excision. A target vector, with the AR exon 2 floxed and selection cassettes in place, was constructed. This target vector was then transfected into mouse embryonic stem (ES) cells using electroporation, where it homologously recombined with the wild type AR locus. The correct homologous recombination was verified using Southern blot analysis and

then transfected with the Cre expression plasmid to excise the floxed neomycin selection cassette. Southern blot analysis was employed again to check that only the selection cassette had been excised and that exon 2 remained floxed. These ES cells were then injected into blastocysts from Swiss-Webster females and implanted into pseudopregnant mothers and left to go to term. Chimeric males were identified and mated to Swiss females to test the germline transmission. Offspring were genotyped using PCR and two chimeric males displaying 100% germline transmission were used as founders to create the heterozygous females. When the Cre males and *loxP* females are mated, male offspring heterozygous for the PGK-Cre allele and inheriting the AR^{flox} from their mother, are likely to undergo exon 2 excision from the early zygote stage onwards, thus generating males with a defective AR (Figure 2.1).

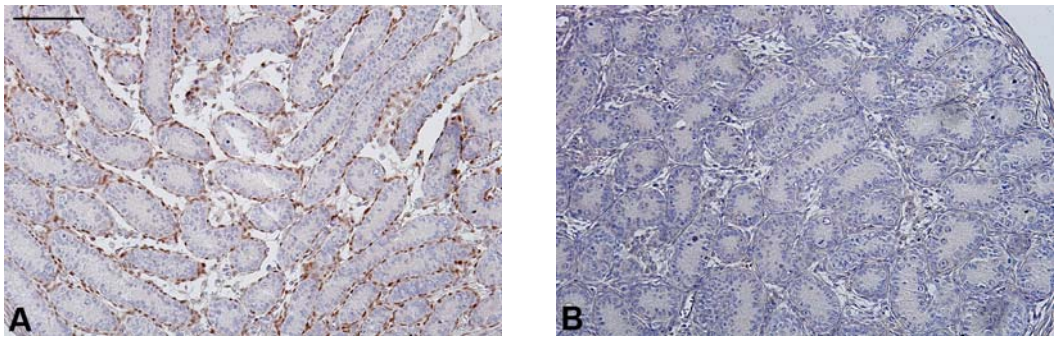

Figure 2.1 Photomicrograph demonstrating AR-positive immunoexpression in the peritubular myoid cells of PGK-Cre mice (A) and the absence of AR expression in the ARKO (B). Scale bar shows 100μm.

The *tfm* mice were a gift from Professor Peter O'Shaughnessy. They have a single base deletion in the coding region of the AR mRNA, located in the amino-terminus domain of the receptor. It is thought that this causes a frame-shift in translation and thus results in the premature termination of AR mRNA synthesis (He et al., 1991). The *tfm* mice were bred in Glasgow on a C3H/HeH-101/H genetic background from stock animals obtained

originally from the Medical Research Council Radiobiology unit (now the Medical Research Council, Mammalian Genetics Unit, Harwell, UK). The mice used in these studies were identified phenotypically, specifically animals that had formed testes but which lacked Wolffian ducts denoting that they were *tfm*.

2.4 Necropsy procedure

Pregnant dams were killed by inhalation of carbon dioxide followed by cervical dislocation under Schedule 1 of the Animal (Scientific Procedures) Act 1986. Once removed from the dam, fetuses were placed in ice-cold 0.01M phosphate buffered saline (PBS, Sigma). Fetuses in late gestation (i.e. e19.5 and older) were decapitated before being placed in ice-cold PBS.

2.4.1 Gross dissection

Once deceased, the pregnant dam was placed on her back, and the abdomen opened. The uterus was removed, each amniotic sac cut into and each umbilical cord severed. Once fetal bodyweight and anogenital distance had been determined, fetuses were place in chilled PBS and transported to the dissection area on ice, to avoid degeneration.

2.4.2 Fetal Bodyweight

Pups taken at e21.5 were gently wiped cleaned and labelled using permanent marker; this enabled the bodyweight, AGD and testis weight for each individual animal to be recorded. Bodyweight was evaluated, using an electronic analytical balance (Handy H110, Sartorius), before the pups were decapitated.

2.4.3 Measurement of anogenital distance (AGD)

The distance between the anus and genital tubercule was measured on e21.5 pups using electronic digital callipers with a resolution of 0.01mm (Faithfull Tools, Kent). Measurements were taken by two independent scientists and averaged. In normal male rats AGD should be 1.5-2 times greater than in female rats from e21.5 onwards. If the AGD of treated males is reduced, this suggests under-masculinisation, as seen in rats treated with anti-androgenic compounds (McIntyre et al., 2001).

2.4.4 Fine dissection

Fine dissection of fetuses took place in a designated dissection area of the laboratory. Testes were removed from fetuses under a binocular dissecting microscope (Leica, MZ6) with a transilluminated stage. Extra lighting was provided by external cold lights (Leica CLS 150x), designed to inflict minimal heat damage on the tissue they illuminate. For stability, fetuses were laid on their backs on a bed made from paper towel dampened with PBS. The abdomen was opened with a wide horizontal incision just below the level of the umbilical cord. The intestines were pushed up and out of the abdominal cavity, to reveal the gonads and urogenital tract. The gonads were located between the kidneys and bladder, depending on gestational age and treatment. Testes were removed and placed in chilled PBS in a petri dish for microdissection.

2.4.5 Microdissection

Testes were trimmed, using the bevelled edge of 27G needles (Monoject, sterile needles, 0.4mm x 12mm) attached to disposable plastic 1 ml syringes, as scalpels to remove the fetal ‘epididymis’ and efferent ducts.

2.4.6 Tissue preservation

One testis from each fetus was snap frozen on dry ice in a 1.5ml Nunc tube. These tubes were clearly labelled and archived at -80°C. The other testis was fixed by immersion in Bouin's solution (BIOS; section 2.12), a formaldehyde based fixative containing picric acid, which forms cross-links between proteins and aldehydes producing a stable structure without losing or damaging antigenic sites. Bouin's is well established for preserving both embryonic and endocrine tissues. Fetal testes were submerged in at least x10 their own volume of Bouin's in an airtight container for 1 hour. Testes were then transferred to 70% ethanol, and weighed using an electronic analytical balance (Handy H110, Sartorius).

2.4.7 Fixed tissue processing

Once tissue had been transferred to 70% ethanol it was processed by the histology support service, MRC Human Reproductive Sciences Unit. In brief, tissue was processed through a series of graded alcohols using the 18 hour automated cycle on a Leica TP-1050 (Leica UK Limited, UK) tissue processor, then embedded into molten paraffin wax by hand. Once cooled the wax blocks were stored at room temperature.

2.5 *In vitro* experiments

Testes from fetuses from untreated dams were removed from e19.5 pups and carefully dissected into explants. They were then cultured for 48 hours with a variety of treatments designed to manipulate Sertoli cell proliferation. The culture method used was developed by Nina Hallmark and combined and adjusted the protocols of Thomson *et al*, 1997, who cultured fetal rat prostates and Robinson *et al*, 2003, who cultured fetal human testes and ovaries (Robinson et al., 2003; Thomson et al., 1997). The culture method derived by

Nina was deemed suitable for studies in this thesis as it was established that testis explants cultured using this method maintained a large portion of normal tissue architecture and also demonstrated a Sertoli cell proliferation index of >30% (Hallmark et al., 2007).

2.5.1 Testis explant preparation

Fetuses were removed and dissected as previously described (2.4). Testes were further dissected into eight pieces to minimise tissue death through oxygen and nutrient depletion. The testis was held in position using a 23G needle attached to a disposable plastic 1ml syringe and halved in line with the direction of the cords, using a scalpel blade (Swann Morton, UK). Each half was then further dissected into four pieces, following the planes of dissection show in Figure 2.2.

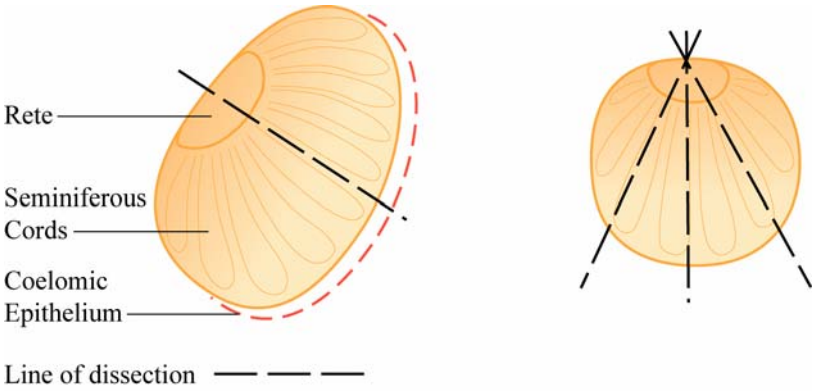

Figure 2.2 Planes of dissection used to create testis explants.

2.5.2 Primary tissue culture conditions

Culture media (Table 2.2) and culture experiments were prepared and performed in a sterile class 2 microbiological safety cabinet (BioMAT-2, MAT, Lancashire, UK). Cultures were incubated (Gallenkamp CO_2 incubator) for 48 hr at 37°C, the temperature of *in situ* fetal testes. The incubator was kept humidified and contained 95% air and 5% CO_2.

Table 2.2 Culture media used with *in vitro* treatments.

Basic Media Type	DMEM/F12	Gibco (UK) Invitrogen Ltd.
Supplements	Sodium Pyruvate	Sigma (Poole, UK)
	ITS liquid media supplement: insulin from bovine pancreas, human transferrin & sodium selenite	Sigma
	Bovine serum albumin (BSA)	Sigma
Anti-microbial products	Penicillin/Streptomycin	Sigma
	Amphotericin	Sigma

2.5.3 Culture set up

Explants were evenly distributed onto the porous membranes of culture well inserts (Millicell 0.4 μm, Millipore, UK) and positioned in a 24-well culture plate (Corning, NY), with a maximum of 5 explants per insert. Pre-warmed (37°C) control/treated media was added, 200μl within the insert, and 200μl within the well, but outwith the insert (Figure 2.3). Two hours prior to the end of the 48 hour culture period the treatment media was removed and replaced with pre-warmed media containing approximately 10 μg/ml 5-Bromo-2'-deoxyuridine (BrdU; in excess), and cultures returned to the incubator. At the end of the culture period testis explants were removed from inserts, fixed in Bouin's for 30 min and then transferred to 70% ethanol. Explants were processed into paraffin wax using the methods described in 2.4.7.

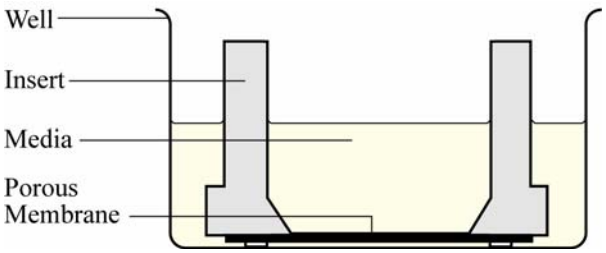

Figure 2.3 Insert positioned in well, 200 µl media was added to the centre of the insert and 200 µl added to the well.

2.5.4 Culture treatments

Table 2.3 Summary of chemical treatments added to *in vitro* fetal testis explants.

Chemical Treatment	Source	Solvent	Target Concentration
Hydroxyflutamide	Toronto Research Chemicals Inc. (Canada)	Media	10^{-4}M, 10^{-5}M, 10^{-6}M
FSH (follicle stimulating hormone)	Organon (UK)	Media	0.05 iu/ml
hCG (human chorionic gonadotrophin)	Serono (USA)	Media	0.1 iu/ml
FSH + hCG	Organon and Serono	Media	0.05 iu/ml & 0.1 iu/ml
EGF (epidermal growth factor)	Sigma	Media	10 nM
Cyclopamine	Gift from Dr William Gaffield, USDA, CA.	Ethanol	25 µM

Explants were exposed to known concentrations of treatment compounds (Table 2.3) via the culture media. Where solvents were necessary, equivalent volumes were added to the media of corresponding controls. All doses used had been previously established for cultures in house.

2.5.4.1 Hydroxyflutamide

Hydroxyflutamide is the active, α-hydroxylated form of flutamide (2.2.2), a synthetic, anti-androgenic compound that competes with testosterone and

DHT for androgen receptor binding (Peets et al., 1974). Since it has been hypothesised that androgens play a role in Sertoli cell proliferation (Atanassova et al., 2005; Johnston et al., 2004; Ramaswamy et al., 2000; Tan et al., 2005), testis explants were exposed to hydroxyflutamide in order to block/minimalise androgen action within the testis explants and thus cause a reduction in Sertoli cell proliferation.

2.5.4.2 Follicle Stimulating Hormone (FSH)

FSH is synthesised in the anterior pituitary and released in response to pulses of gonadotrophin releasing hormone (GnRH) from the hypothalamus. It is already well established that FSH stimulates Sertoli cell proliferation *in vivo* (Allan et al., 2004; Johnston et al., 2004; Meachem et al., 1996), so FSH was utilised in these studies to test the sensitivity of the Sertoli cells to FSH *in vitro* and also to test the reliability of the *in vitro* system.

2.5.4.3 human Chorionic Gonadotrophin (hCG)

Chorionic Gonadotrophin is a glycoprotein hormone produced by the trophoblastic cells of the developing placenta, where its role is to maintain the corpus luteum. It is structurally very similar to luteinising hormone (LH), sharing the same α-subunit, and it is because of this similarity that hCG can be used clinically to activate a cascade of responses at the LH-receptor and induce greater levels of testosterone production than equivalent amounts of LH (Zhou and Hutson, 1995). hCG is not normally expressed by the rat placenta, so the commercially available product used for these experiments was human derived (Serono). hCG was used in these experiments to stimulate endogenous testosterone production (Hallmark et al., 2007) to investigate whether this would increase Sertoli cell proliferation.

2.5.4.4 Epidermal Growth Factor (EGF)

EGF is known to be important in cell growth, proliferation and differentiation and has been shown to play a role in reproductive tract development (Gupta et al., 1991). It has also been shown to stimulate Sertoli cell proliferation *in vitro* in postnatal testes (Petersen et al., 2001). For this reason EGF was added to cultures in order to ascertain whether or not it could stimulate Sertoli cell proliferation in fetal testes.

2.5.4.5 Cyclopamine

Cyclopamine is a naturally found steroidal alkaloid that inhibits the hedgehog signalling pathway and thus blocks the cell's response to the hedgehog proteins. Smoothened protein is a GPCR, important in the hedgehog signalling pathway. In un-stimulated cells, Smoothened is repressed by Patched, the receptor for hedgehog ligands. When Patched is activated by a hedgehog ligand, it activates Smoothened which stimulates transcription factors to induce the expression of specific genes. Cyclopamine binds to and blocks the Smoothened protein, thus inhibiting the hedgehog pathway (reviewed; (King, 2002).

Desert hedgehog (Dhh) is expressed in Sertoli cells (Bitgood et al., 1996) and its receptor, Patched 1 (PTCH1), is expressed in Leydig cells and peritubular myoid cells (Clark et al., 2000). It has been shown that inhibition of the Dhh/PTCH1 signalling pathway results in disrupted cord formation (Yao and Capel, 2002), incomplete Leydig cell differentiation (Yao et al., 2002) and in mice lacking Dhh, 92.5% of the males were under-masculinised, with abnormal peritubular myoid cells and severely restricted spermatogenesis (Clark et al., 2000). Cyclopamine was used in these experiments to investigate the involvement of Dhh signalling between Sertoli cells and

peritubular myoid cells and any effect on Sertoli cell proliferation, as well as to examine the effect that disrupting Leydig cell differentiation may have on Sertoli cell proliferation.

2.5.5 Culture contamination

Cultures were at risk of contamination from a variety of microorganisms, so sterile techniques were employed wherever possible, although this was difficult when retrieving testes from animals. Media preparation and culture set-up were carried out in a safety cabinet and gloves were worn at all times. Anti-microbial agents were added to the media; these chemicals were routinely used in cultures in-house and have not been shown to have any adverse affects on cultured tissue. The agents used were Penicillin/Streptomycin (Sigma; antibiotics) and Amphotericin (Sigma; antifungal).

2.6 Protein investigations

2.6.1 Immunohistochemistry

Antibodies were used to detect protein expression within sections of Bouin's fixed tissue. The basic protocol followed is outlined below:

- Tissue dewaxed and re-hydrated
- Retrieval of target antigen for detection by specific primary antibody
- Blocking of non-specific antigens
- Incubation with primary antibody
- Detection of primary antibody using amplification system
- Visualisation of antibody using colour reaction
- Counterstaining of non-stained tissue

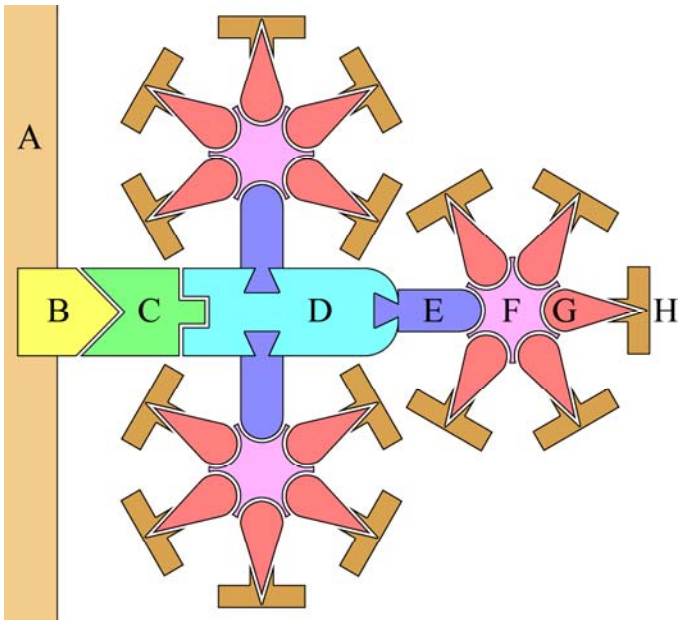

Figure 2.4 Schematic representation of the principles of direct Immunohistochemistry. Within the cell (A), proteins are expressed which have antigens (B). These antigens are recognised by specific primary antibodies (C), to which the secondary antibody (D) binds, with its biotin conjugates (E). An avidin-biotin-enzyme complex (F) attaches to the conjugated biotin and the enzyme (G) reacts with the chromogenic substrate to create a visible colour change (H) at the site of the antigen expression. The indirect immunohistochemistry method replaces D, E, F and G with a single complex, for example, Envision (DAKO).

2.6.1.1 Sectioning

Wax blocks containing fixed tissue were chilled on ice prior to sectioning; this hardened the wax making it easier to cut. 5µm thick sections were cut using a hand-operated microtome (RM 2135, Leica), then floated onto 30% industrial methylated spirit (IMS, Fischer Scientific), followed by warm (45-50°C) water, to smooth out any wrinkles in the wax or section. Individual sections were then mounted onto pre-labelled (automated slide labeller, Leica IP S), charged glass slides (SuperFrost, Menzel GmbH & Co.) and stacked in a metal rack. Slides were allowed to dry overnight at 50°C.

2.6.1.2 Dewaxing and re-hydration

Wax was removed from the slides by submerging them in Xylene, for 5 minutes, x2. The slides were then re-hydrated through a series of alcohols, spending 20 seconds in each. They passed from absolute alcohol (x2), to 95% alcohol, 80%, then finally to 70% alcohol. Slides were then washed in tap water.

2.6.1.3 Antigen retrieval

Fixation with formaldehyde based fixatives such as Bouin's causes the formation of protein cross-links that can mask the antigenic sites within a tissue. Antigen retrieval breaks these cross-links allowing the immuno-detection of previously hidden antigens (Shi et al., 1993). Antigen retrieval was not required for the detection of all antigens. Once dewaxed and re-hydrated, slides were immersed in 2L of boiling retrieval buffer, usually 0.01 M citrate buffer (pH 6.0; section 2.12), contained in a Tefal Clipso pressure cooker (Tefal). The lid was sealed and the pressure set at the highest setting. Once full pressure was achieved, as depicted by the onset of a continuous jet of steam, the slides were left for 5 minutes. After this time the pressure was released and the pressure cooker removed from the heat. The buffer was left to cool for 20 minutes, after which the slides were removed, cooled in H_2O and then transferred to Tris buffered saline (TBS; section 2.12).

2.6.1.4 Blocking

Slides were incubated in 3% (vol/vol) hydrogen peroxide (BDH) in methanol for 30 minutes to block any endogenous peroxidase activity, which could cause non-specific background staining. Slides were then rinsed in tap water and washed twice, in TBS, for five minutes. Once washed, the excess TBS was wiped from around each tissue sample and the sections then drawn

around with ImmEdge (Vector Laboratories, Inc. Burlingame, CA), a hydrophobic barrier pen that allowed clearer visualisation of small sections and provided a water-repellent barrier to ensure maximum coverage of sections with staining reagents.

Slides were blocked in normal serum (Diagnostics Scotland) diluted 1:5 in TBS containing 5% bovine serum albumin (BSA, Sigma) to block non-specific binding sites. The type of serum used depended on what species the secondary antibody was raised in. For example, goat serum would be used for blocking with a goat raised anti-rabbit secondary.

2.6.1.5 Primary antibodies

The blocking serum was replaced with the appropriate primary antibody (Table 2.4), diluted as necessary in normal serum/TBS/BSA, and incubated overnight at 4°C in a humidity chamber.

Table 2.4 Details of primary antibodies used.

Antibody	Source	Retrieval	Species	Dilution
AMH (anti-mullerian hormone)	Santa Cruz, CA	None	Goat	1:1000
AR (androgen receptor)	Novocastra, Newcastle-upon-Tyne, UK	Citrate	Rabbit	1:20
Desmin	DAKO, Cambridgeshire, UK	Citrate	Mouse	1:500
DMRT1	Gift from David Zarkower (Minnesota)	Citrate	Rabbit	1:2000
Glucocorticoid receptor	Santa Cruz, CA	Citrate	Rabbit	1:200
SMA (smooth muscle actin)	Sigma, Poole, UK	None	Mouse	1:10 000
Pancytokeratin	Sigma, Poole, UK	Citrate	Mouse	1:400
Vimentin	DAKO, Cambridgeshire, UK	Citrate	Mouse	1:1000
WT1 (Wilms tumour gene 1)	DAKO, Cambridgeshire, UK	Citrate	Mouse	1:1000
BrdU (5-Bromo-2'-deoxyuridine)	Roche Diagnostics Corporation, IN, USA	Citrate	Mouse	1:2000
3-beta hydroxysteroid dehydrogenase (3-β HSD)	Gift from Ian Mason (Edinburgh)	None	Rabbit	1:4000
Laminin	Abcam, Cambridge, UK	Citrate	Rabbit	1:200
P75 neurotrophin receptor (P75NTR)	Abcam, Cambridge, UK	None	Rabbit	1:50
Nestin	Abcam, Cambridge, UK	Citrate	Mouse	1:50

2.6.1.6 Secondary antibodies (Direct method)

The direct method of amplification was a three-step process. After incubation with the primary antibody, slides were washed in TBS for five minutes, twice. The appropriate secondary antibody, conjugated to biotin, was diluted in blocking serum, added to the slides and incubated for 30 minutes at room temperature. The secondary antibody was then removed with two TBS washes, as before.

Table 2.5 Details of secondary antibodies used.

Antibody	Source	Dilution
Rabbit anti mouse biotinylated	DAKO (Cambridgeshire, UK)	1:500
Goat anti rabbit biotinylated	DAKO (Cambridgeshire, UK)	1:500

The biotinylated secondary antibody signal was then amplified using an avidin-biotin labelled horseradish peroxide enzyme conjugate (ABC-HRP). This was prepared 20 minutes before use by adding one drop of solution A (DAKO) and one drop of solution B (DAKO) to 5ml of Tris-HCl. In later studies, ABC-HRP was replaced with streptavidin-HRP (DAKO), as the streptavidin is less likely to bind non-specifically. Streptavidin-HRP was prepared by adding 1μl solution to 1ml TBS. The final step involved exposing the slides to a chromogenic substrate (2.6.1.8) so that a colour change reaction occurred in the specific regions where the primary antibody had bound (Figure 2.4).

2.6.1.7 Double stain procedure (Direct and Indirect methods)

This procedure was employed to detect two primary antibodies (3-β HSD and SMA; Table 2.4) simultaneously. Slides were dewaxed and re-hydrated (2.6.1.2) and then blocked in methanol peroxidise (2.6.1.4); antigen retrieval

was not required for either of these primary antibodies. Slides were blocked with normal goat serum (2.6.1.4) before incubation overnight with the 3-β HSD primary antibody (Table 2.4). Slides were washed twice with TBS for 5 minutes, then incubated with goat anti rabbit biotinylated secondary antibody for 30 minutes. Slides were washed as before with TBS, then exposed to an avidin-biotin labelled alkaline phosphatase enzyme conjugate for 30 minutes. This was prepared 20 minutes before use by adding one drop of solution A (DAKO) and one drop of solution B (DAKO) to 5ml of Tris-HCl. The localisation of the primary antibody was visualised using Fast Blue chromogen detection (2.6.1.8). Slides were washed twice in TBS, and then re-blocked in normal goat serum for 30 minutes, before overnight incubation with the SMA primary antibody (Table 2.4). This time the indirect method of amplification was employed, whereby the secondary antibody and avidin-biotin complex (ABC) were replaced with a single step. Envision anti mouse (DAKO), a secondary antibody polymer that already had the HRP labelled polymer bound, was applied to the slides, straight from the bottle, for 30 minutes. The removal of ABC reduces the risk of non-specific binding and makes this method of amplification far more sensitive. Once the Envision was removed with two TBS washes, the SMA primary antibody was visualised, this time using DAB (3,3 DiAminoBenzidine) chromogen detection (2.6.1.8).

2.6.1.8 Chromogen detection

In order to visualise the primary antibody and thus determine where in the tissue the specific protein was located, two chromogen substrates were used. Each substrate formed a different coloured precipitate, so could therefore be used together, in a double stain procedure.

Brown: DAB (3,3 DiAminoBenzidine) was bought in a concentrated form (DAKO) and diluted just prior to use. Once applied to the slides, it usually took between 30 seconds and five minutes to develop, depending on the primary antibody used. Once optimum staining (in controls) had occurred, slides were immersed in tap water to stop the reaction.

Blue: Fast Blue was made in house by adding 1mg fast blue salt (Sigma) to 1ml fast blue buffer (section 2.12). The solution was filtered through a 0.2µm filter directly onto the slides. Fast Blue usually took around 20 minutes to develop and once staining was optimum, the reaction was stopped with tap water. Fast Blue staining was used as the second stain in double stain procedures and due to its colour (blue) was not counterstained with haematoxylin.

2.6.1.9 Counterstaining: Haematoxylin, Toludine Blue and Periodic Acid Schiff

Slides were counterstained with haematoxylin (section 2.12), for four minutes if they had not been antigen retrieved in Citrate, and six minutes if they had, this over-exposure ensured the saturation of all binding sites. Slides were then rinsed in tap water before being briefly immersed in 1% acid alcohol (section 2.12), to remove non-specific background staining, then rinsed again in tap water. The slides were then immersed in Scott's tap water (section 2.12), an alkaline solution, for approximately 30 seconds. The nuclear stain turned blue in the alkaline environment. Slides were then rinsed in tap water.

Slides required for multinucleated gonocyte analysis (2.7.5) were stained with toluidine blue, as this enabled clearer visualisation of the gonocytes. Slides were dewaxed and re-hydrated as before (2.7.1.2). Toluidine blue stain

(1% toluidine blue, 1% di-Sodium tetraborate, BDH chemicals) was filtered and applied to the slides at a 50% dilution in distilled water for 10 seconds. Slides were then rinsed in tap water.

Periodic acid-schiff (PAS) staining was used to stain the basal lamina. The periodic acid oxidises the glucose residues in the basal lamina, creating aldehydes that react with the Schiff reagent to form a magenta-coloured compound. Slides were dewaxed and re-hydrated. They were immersed in periodic acid (1% aqueous, BIOS) for 5 minutes, rinsed in distilled water, then submerged in Schiff's solution (Feulgen stain, BIOS) for 15 minutes. Slides were then placed under running tap water for 10 minutes, allowing the stain to develop pink. Slides were then counterstained with haematoxylin (see above).

2.6.1.10 Dehydration and mounting

Slides stained with DAB (2.6.1.8), haematoxylin or Toludine blue, were dehydrated through increasing concentrations of alcohol, from 70% to absolute alcohol, and then cleared in Xylene for 5 minutes x2. These slides were then mounted beneath glass coverslips (VWR International) using Pertex mounting medium (Cell Path, Hemel Hampstead, UK), a solvent based glue.

Slides stained with PAS were dehydrated in only two absolute alcohols, before being cleared in xylene and mounted with pertex, as dehydration through all of the alcohols resulted in the loss of some of the PAS staining.

Slides visualised with fast blue (2.6.1.8) were not dehydrated, because Fast Blue is soluble in alcohol and would therefore be removed. Consequently, after counterstaining, slides were rinsed in water then mounted under glass coverslips using Permafluor (Beckham Coulter), an aqeous mounting

medium. The disadvantages of mounting with Permaflour however, was that air bubbles were far more frequent and coverslips were not as firmly fixed to the slide as with Pertex.

2.6.1.11 Imaging

Images were examined using a Provis microscope (Olympus Optical., London, UK) and photographed using a DCS330 digital camera (Eastman Kodak, Rochester, NY). Captured images were compiled using Photoshop 7.0 (Adobe Systems Inc, Mountain View, CA).

2.7 Image analysis

Image-Pro Plus 4.5 (Media Cybernetics, Wokingham, Berkshire, UK) computer software, combined with Stereology-pro-5.0 software (Media Cybernetics, Wokingham, Berkshire, UK) and a microscope with camera and motorised stage (Prior Scientific Instruments Ltd., Cambridge, UK), were used to quantify the number of Sertoli and peritubular myoid cells, the intensity of SMA staining in the peritubular myoid cells compared to an internal control and the distribution and clustering of fetal Leydig cells.

2.7.1 Determination of Sertoli cell number per testis

Standard stereological techniques were used to establish whether Sertoli cell number per testis was altered after treatment. Cross-sections of fetal testes were immunostained for WT1 to label Sertoli cell nuclei, and were then examined under oil immersion using a Leitz ×63 plan apo objective fitted to a Leitz laborlux microscope and a 121-point eyepiece graticule. Fifteen fields were selected and counted using a systematic clock-face sampling pattern from a random starting point; points falling over Sertoli cell nuclei were scored and expressed as a percentage of the total points counted, or

percentage volume occupancy. For each animal, the values for percentage nuclear volume were converted to absolute nuclear volumes per testis by multiplying by testis weight (see section 2.7.1.1), which is equivalent to volume, because shrinkage was minimal.

Absolute volume (mg) = (% volume occupancy/100) x testis weight (mg)

Sertoli cell nuclear volume per testis equates to cell numbers per testis, assuming no change in average nuclear size. To verify that treatment had not induced a change in average nuclear volume, this was assessed in each animal, using methods similar to those described previously (Atanassova et al., 1999; Sharpe et al., 2000). Images were analysed at x63 magnification with Image-Pro Plus 4.5.1 software with a Stereology 5.0 plug-in (Media Cybernetics, Wokingham, Berkshire, UK). The Selector tool was used to denote the centre of the Sertoli cell nucleus, and then three diameter measurements (μm), all passing through the centre of the nucleus, were made. The nuclear volume of each Sertoli cell was calculated by the software, which assumed that the nuclei were spherical. Fetal Sertoli cells actually have irregularly shaped nuclei, but this assumption was made for all Sertoli nuclei, ensuring that any errors were consistent. The nuclear volume of at least 90 Sertoli cell nuclei was measured per testis, and the mean nuclear volume then determined. Data for Sertoli cell nuclear volume per testis was then converted to absolute numbers of Sertoli cell per testis by dividing by the average Sertoli cell nuclear volume:

$$\text{Number of cells (millions)} = \frac{\text{Absolute volume (mg)}}{\text{mean nuclear volume } (\mu m^3)} \times 1000 \text{ (to account for mg to } \mu m^3)$$

Other experiments within this laboratory have demonstrated that this method of determining Sertoli cell number is directly comparable with results obtained using the 'unbiased' method (optical disector) e.g.

Atanassova *et al* (2005). It was therefore deemed more appropriate for these studies since a) the testes used were very small and dissecting them into transverse sections for the disector method would have been difficult, and b) sections from each testis were also required for other forms of analysis, so fixing and processing them as standard paraffin blocks and cutting them into 5μm sections meant we were able to reduce the number of animals used for these studies.

2.7.1.1 Calculating volume (weight) of e17.5 testes

Testes taken from e17.5 pups were too small to be weighed using the electronic analytical balance (Handy H110, Sartorius). In order to calculate an average weight/volume, three control and three DBP-treated e17.5 Bouin's-fixed testes were serially sectioned (see section 2.6.1.1). The first section, every fifth section and the final section were stained with Haematoxylin for clear visualisation. The area of each of these sections was then measured using the Image-Pro Plus 4.5.1 software at x10 magnification. The areas of adjacent sections (e.g. sections A and B) were averaged and then multiplied by the distance between them (distance C), to give the volume. This was done for all sections, giving an overall volume (μm^3), equal to weight (mg).

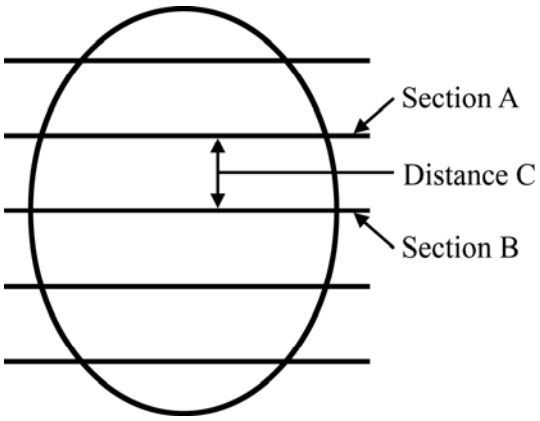

Figure 2.5 Representation of how testis volume was calculated.

2.7.2 Determination of peritubular myoid cell number per testis

Peritubular myoid cell number per testis was determined using similar procedures as used for Sertoli cell number (2.7.1), with the exception of three changes. Firstly, due to the smaller size and lower frequency of peritubular myoid cells, 50 fields were selected to determine percentage volume occupancy. Secondly, because the nuclei of the peritubular myoid cells are ellipsoid in shape, the mean nuclear volume was calculated using a different method (see section 2.7.2.1), and thirdly, sections were immunostained with AR to stain the peritubular myoid cell nuclei.

2.7.2.1 Calculation of the mean nuclear volume of peritubular myoid cells

The nuclei of the peritubular myoid cells are oblate ellipsoids. The formula for calculating the volume of an ellipsoid is:

$$\text{Volume} = 4/3\pi \times \text{height} \times \text{width} \times \text{length}$$

An oblate ellipsoid is defined as having $\text{length} = \text{width} < \text{height}$. Consequently, Image-Pro Plus 4.5.1 software was used to measure the length and height of the Peritubular myoid cell nuclei, and the width, which could not be measured, was assumed to be equal to the length. Using the formula above, the volume was calculated for each of the 90 Peritubular myoid cell nuclei counted per animal. Mean nuclear volume was then calculated and used to determine Peritubular myoid cell number per testis as in section 2.7.1.

2.7.3 Cell proliferation index (PI)

The PI represents the percentage of cells within a certain population (e.g. Sertoli cells) that were positively stained for the proliferation marker BrdU (see section 2.2.6). When calculating the proliferation index of Sertoli cells in

cultured testis explants, BrdU-positive and BrdU-negative Sertoli cells were counted in all complete seminiferous cords (cords around the edges of the explants were often apoptotic and as such malformed/incomplete). These counts were made by tagging a captured live image (at x63 magnification) and tracked per cord on a second screen using a tiled image (3x3 frames) taken at x20. When calculating the proliferation index of Sertoli cells in sections of testes that had been treated *in utero*, BrdU-positive and BrdU-negative Sertoli cells were counted in a minimum of 15 cords. Fifteen fields were selected at random by the software, and all the Sertoli cells (BrdU-positive and BrdU-negative) located in seminiferous cords in each of those fields was counted, using the tagging and tracking system described above. The PI was calculated using the following formula:

$$\text{Proliferation Index} = \frac{\text{Number Sertoli cells expressing BrdU}}{\text{Total number Sertoli cells}} \times 100$$

2.7.4 Determination of Leydig cell aggregation

As *in utero* exposure to DBP has previously been shown to induce Leydig cell aggregation in fetal testes (Mahood et al., 2005; Mahood et al., 2006), and also causes a reduction in testosterone levels (Shultz et al., 2001), this end point was investigated further to determine whether reduced androgen action plays a role in inducing fetal Leydig cell aggregation. To determine whether the distribution of Leydig cells throughout the fetal testis was altered by treatment, Leydig cell aggregation was quantified by analysing the number and size of Leydig cell clusters using methods previously described in (Mahood et al., 2005). Testes were serially sectioned and three representative sections from each testis then selected and immunostained for 3β-HSD. The three sections chosen were those corresponding to ~25, 50 and 75% intervals through the serially sectioned testis; at e21.5 in rats this corresponded to

sections that were 20-30 sections apart from each other. Testis sections from at least six males from a minimum of three separate litters from each treatment group were used for analysis. Quantification of Leydig cell cluster number and area in these sections was undertaken using Image-Pro Plus 4.5.1 software. Sections of testis immunostained for 3β-HSD (but not counterstained) were of sufficient homogeneity, high contrast, and low background to allow computer-assisted thresholding and subsequent computer-assisted counting of Leydig cell (3β-HSD-immunopositive) clusters and determination of Leydig cell cluster area. Digital images of complete testis sections were captured at ×40 magnification. The software was used to trace around each section, creating the area of interest to be analysed. Computer-assisted thresholding was then used to identify and analyse clusters of 3β-HSD-immunopositive cells, generating data on the number of clusters, the total area of clusters, and the area occupied by each Leydig cell cluster (Figure 2.6).

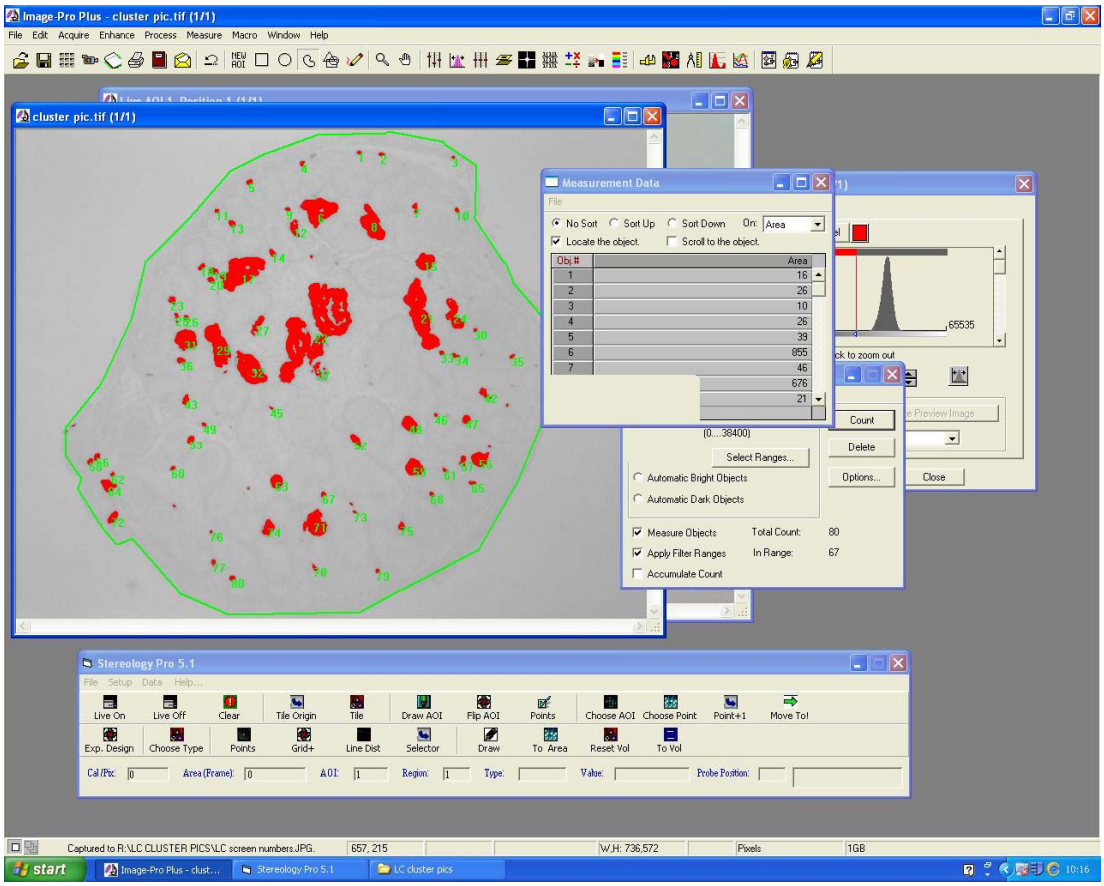

Figure 2.6 Captured screen image showing the Leydig cell clusters in red, defined using thresholding. The number of clusters is recorded, as is the area of each cluster.

Each cluster was then expressed as a percentage of the total Leydig cell cluster area (100%) in that animal and was then assigned to one of three groups: small clusters, which accounted for 5% or less of the total Leydig cell cluster area per testis; medium clusters, that accounted for 5.1% to 14.9%; and large clusters, which accounted for 15% or more of the total Leydig cell cluster area per testis. This approach was necessary to take account of the reduction in total Leydig cell area per testis in DBP-exposed animals due to reduction in Leydig cell size (Mahood et al., 2005).

2.7.5 Analysis of multinucleated gonocyte frequency

In utero exposure to DBP has previously been shown to induce multinucleated gonocytes (Fisher et al., 2003; Parks et al., 2000), as well as cause a reduction in intratesticular testosterone (Shultz et al., 2001). Analysis of multinucleated gonocyte frequency after *in utero* exposure to a variety of treatments allowed further investigation into the role that androgens play in testicular dysgenesis. In order to do this representative testis sections were stained with toluidine blue (see section 2.6.1.9) to enable clear visualisation of the gonocytes. Stereological analysis of the occurrence of multinucleated gonocytes was performed using Image-Pro Plus 4.5.1 software. Each seminiferous cord was viewed at x63 magnification, and each cord tagged on a second screen using a tiled image (3x3 frames) taken at x20 in order to keep track. Cords containing one or more multinucleated gonocyte were tagged in a different colour to enable the occurrence of multinucleated gonocytes (MNG) to be calculated and expressed as a percentage:

$$\%\ \text{seminiferous cords containing MNG} = \frac{\text{Cords containing} \geq 1\ \text{MNG}}{\text{Total number of cords}} \times 100$$

2.7.6 SMA quantification

It has been suggested that expression of SMA in the peritubular myoid cells denotes the differentiation of these cells, and that this differentiation is, in part driven by androgens (Schlatt et al., 1993). To determine whether the intensity of SMA staining in the peritubular myoid cells was altered following treatment, representative testis sections were immunostained for SMA. Quantification of the intensity of SMA staining was determined using Image-Pro Plus 4.5.1 software. Each section was viewed under a Leitz ×20 objective and the range of pixels that constituted SMA staining selected using the eyedropper tool to determine the colours associated with SMA staining.

An area of interest was then drawn around a blood vessel or one of ten randomly selected seminiferous cords, and the software used to measure the mean value of the pixels in that area of staining, giving a measurement of brightness. The intensity of blood vessel staining was used as the internal control and its intensity value adjusted to 100%. The values for the peritubular myoid cell staining were adjusted accordingly to give intensity values relative to the internal control and were expressed as a percentage.

2.8 Hormone analysis

Endocrine effects of *in utero* treatments were quantified by measuring levels of testosterone in whole testis homogenates. A protocol for this hormone assay was already established in-house.

2.8.1 Testosterone assay

2.8.1.1 Extraction of testosterone from whole testis

Each whole fetal testis (a minimum of six testes at e15.5 were pooled to obtain detectable levels of hormone) was homogenized in 0.5ml PBS buffer. The homogenate then underwent an extraction step to solubilise any testosterone in the tissue, including its freeing from proteins such as albumin. 100µl aliquots of homogenate were placed in glass tubes and vortexed for 5 minutes with 2ml di-ethyl ether. The tubes were then sat in a metal bath of dry ice and methanol to freeze the aqueous portion, so that the unfrozen organic portion containing the extracted testosterone could be decanted into clean tubes. These tubes were left overnight in a fume hood to enable the organic solvent to evaporate. The dry residue was then re-suspended in testosterone assay buffer and stored at -20°C until quantified.

2.8.1.2 Testosterone Radioimmunoassay (RIA)

This fluid phase technique relies on competition between the known concentration of radiolabelled antigen (testosterone labelled with I^{125}, APB Biotech, UK) against unlabelled endogenous testosterone present in the sample. The more testosterone in the test sample, the lower the level of binding by the labelled testosterone. Radiolabelled testosterone was added to each sample prior to the addition of primary antibody 505, used at 1:35000. The primary antibody was incubated for 3 hours at room temperature. The samples were then incubated overnight at 4°C with a secondary antibody, donkey-anti-sheep DAGS. The secondary antibody binds to the testosterone/antibody complex to form a stable precipitate. Then 1ml of wash buffer was added and the tubes centrifuged for 30 minutes at 3000rpm. The supernatant was then decanted and the amount of radiation in the precipitate was measured and the test samples compared to a standard curve of known testosterone concentrations. A gamma counter (Multigamma 1261, LKB Wallac, Turku, Finland) was used to measure residual I^{125}. This assay is a precise and sensitive method of measuring testosterone and has a low intra-assay coefficient of variance (<10%). In each run, method blanks, quality control samples (spiked buffer), and standards were analysed alongside the unknown samples. Results were analysed with AssayZap (Biosoft, Cambridge, UK) and data expressed as pg testosterone per 100ul. For presentation purposes, data was converted to ng/ml (=pg/100µl x 0.01), ng/testis or ng/unit testis weight.

Not all testicular extracts were assayed together. To avoid consequent inter-assay errors, testosterone levels for testes from treated animals were expressed as a percentage of the mean value for control testes run in the

same assay. Although I ran several testosterone assays, some results were also generated by Nancy Evans, from the Assay lab, HRSU, Edinburgh.

2.9 Quantitative RT-PCR

Quantitative RT-PCR was carried out using the TaqMan analysis system devised by Applied Biosystems. Primers were designed to each DNA target using the Roche assay design centre, which also provided details of which probe, from the Universal ProbeLibrary (Roche), would bind between the forward and reverse primers chosen.

2.10 Principles of the TaqMan reaction

A probe from the Universal ProbeLibrary (Roche) was assigned to each reaction, so that its location on the target sequence is between the forward and reverse primers. Each probe has a reporter dye, which in this study was 6-carboxyfluorescein (FAM), at the 5' end of the probe, and a quencher dye, 6-carboxy-tetramethyl-rhodamine (TAMRA), at the 3' end. When the probe is intact, the quencher suppresses the fluorescence of the reporter (Figure 2.7).

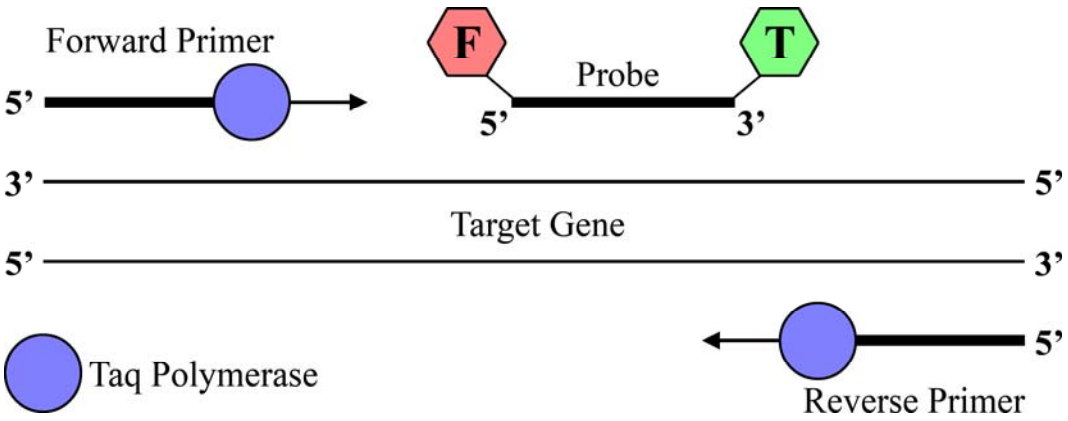

Figure 2.7 Reporter-quencher proximity results in suppression of fluorescence.

If the target sequence is present in the sample being amplified, the probe anneals to the target gene cDNA between the forward and reverse primers.

During amplification the Taq polymerases acts in a 5′ to 3′ direction and cleaves the probe so that the FAM dye is released and no longer quenched by the TAMRA dye (Figure 2.8). This process occurs with every cycle, so fluorescent activity increases every time the probe is cleaved, and this is proportional to the amount of PCR product that is formed. This is measured quantitatively by the sequence detection system.

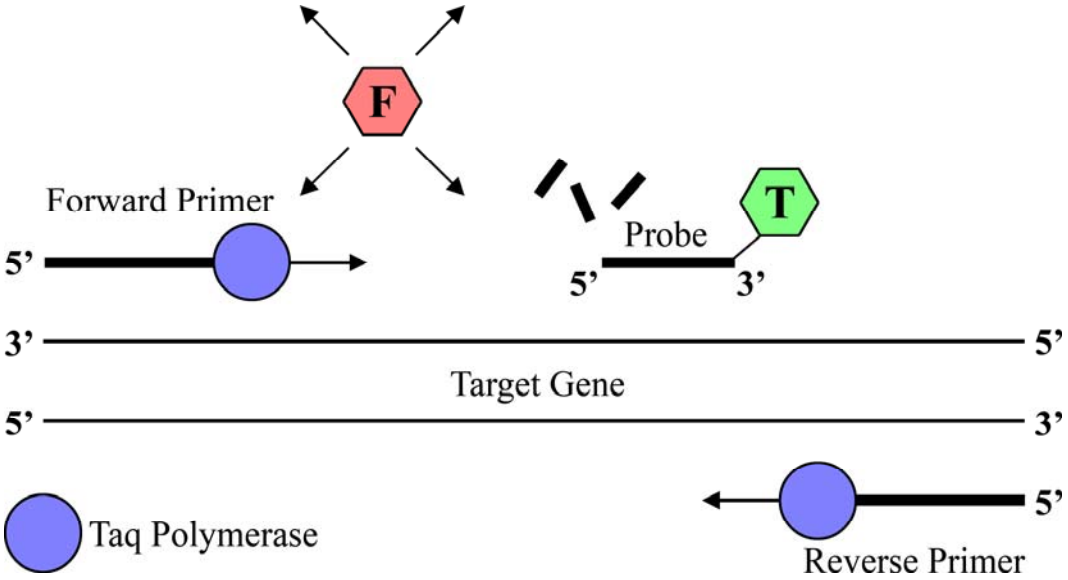

Figure 2.8 Cleavage of the reporter dye from the quencher results in fluorescence.

2.10.1 Primer Design

Primers were designed using the free assay design centre at www.universalprobelibrary.com. This free-access web-based Probe-Finder software designs the primer sequence and tells you what number probe from the Universal ProbeLibrary is compatible. The primers were then purchased from MWG Biotech, London, UK.

Table 2.6 TaqMan primer sequences and Universal ProbeLibrary probe number.

Gene	Forward Primer 5' → 3'	Reverse Primer 3' → 5'	Universal ProbeLibrary, Probe number:
DAX1	CAGGCCATCAAG AGTTTCTTTT	CTCGATGTATTTCA CGCACTG	67
Dhh	ACCGCCTGATGAC AGAGC	GCCTTCGTAGTGCA GTGAGTC	49
DMRT1	CAGAAGCCAAAG CAAGTGTG	AGCTGCTGGAGAGG GAAAC	129
FGF9	CGGTACTATCCAG GGAACCA	CAGGCCCACTGCTA TACTGAT	114
FMR1	CGCGGTCCTGGAT ATACTTC	TGGAGCTAATGACC AATCACTG	114

2.10.2 RNA extraction

RNA was extracted from frozen testes using the RNeasy mini extraction kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. All tubes and instruments used were RNase free. In brief, frozen testes were homogenized in 2ml tubes with steel beads and 350µl RLT lysis buffer, using the Qiagen TissueLyser, set at 20Hz for 30 seconds (e15.5 testes were pooled to increase the yield of RNA extracted). The tissue lysate was centrifuged for 3 minutes at maximum speed (13,000 rpm), and the supernatant mixed with 350µl ethanol. Samples were then transferred onto RNeasy mini columns positioned in 2ml collection tubes, centrifuged for 15 seconds at 10,000 rpm and the flow-through discarded. In order to wash away contaminants, 700µl RW1 wash buffer was added to each column, spun at 10,000 rpm for 15 seconds and the flow-through discarded. This process was repeated twice using 500µl RPE wash buffer, and the second time the columns were spun for 2 minutes to allow the silica-gel membrane to dry. The RNA was then eluted by adding 30µl RNase-free water directly on to the membrane and spinning

at 10,000 rpm for 1 minute. RNA concentrations were measured using the Nanodrop spectrophotometer (section 2.9.2). RNA was stored at -80°C before use.

2.10.2.1 RNA quantification

The concentration and purity of RNA samples was analysed using the Nanodrop-1000 spectrophotometer (Nanodrop Technologies, Delaware, USA). The Nanodrop only requires 1.5µl of sample and this is pipetted directly onto the measurement pedestal. The amount of 260nM UV light absorbed by the sample is measured and the Beer-Lambert law is used to relate this to the concentration of the light absorbing molecule, i.e. the RNA.

The Nanodrop also measures the absorption of light at 280nM, the wavelength absorbed by impurities such as protein, salt and solvents. Given the ratio of 260nM:280nM, the purity of the RNA sample can be ascertained. Ideally the 260nM:280nM ratio should be 2, although values in the range of 1.9 – 2.1 are deemed acceptable.

2.10.3 Preparation of cDNA for TaqMan analysis

Random hexamer primed cDNA was synthesised using the Applied Biosystems TaqMan reverse transcription reagents kit (Applied Biosystems, Foster City, CA). Random hexamer primers, and not oligo dT primers, were used because they amplify total RNA, including ribosomal RNA. This is important for these Taqman reactions because 18s is a ribosomal RNA and was used as the internal positive control.

cDNA was synthesised according to the following reaction:

10x PCR Buffer II	2µl
25mM MgCl2	4µl
dNTP's	2µl
RNase inhibitor	1µl
Random Hexamers	1µl
Multiscribe RT	1µl
RNA (100ng/µl)	4µl
Nuclease free H_2O	5µl

The reagents were mixed together in 0.2ml sterile thin walled PCR tubes (Continental Lab Products, Oxford, UK) and placed in a thermo-cycler with the following cycle times:

25°C for 20 minutes

42°C for 60 minutes

95°C for 5 minutes

2.10.4 Q-RT-PCR reaction

Primers were diluted to 20µM with nuclease free water. Samples required for analysis were run in triplicate on a 96 well MicroAmp optical reaction plate (Applied Biosystems). For each sample, a tube containing the following was prepared:

2x Faststart TaqMan Probe Master (Roche)	30µl
Rox dye* (6µM; Roche)	4.5µl
Forward primer (20µM)	0.6µl
Reverse primer (20µM)	0.6µl
Universal Probe (10µM; Roche)	0.3µl
18s (Applied Biosystems)	17.1µl
cDNA (section 2.9.4)	6µl

* The 2x Faststart TaqMan Probe Master already contains Rox dye at 120nM but this must be supplemented for use on the ABI 7900 machine (Applied Biosystems).

Once combined, the samples were divided into three wells, with 20µl in each. The plate was sealed with an ABI prism optical adhesive cover (Applied Biosystems) and loaded onto the ABI 7900 sequence detection system.

2.10.5 Analysis of results – comparative Ct method

Taqman RT-PCR results are given as an amplification plot, which depicts the amount of reporter dye generated during amplification, and is directly linked to the amount of PCR product formed. An example of an amplification plot is shown in Figure 2.9. The FAM Ct value corresponds to the cycle number at which the fluorescence, representing the amount of amplified target, becomes significant above the background level (the threshold level). The threshold level is determined at the point when an increase in signal is associated with an exponential increase of PCR product.

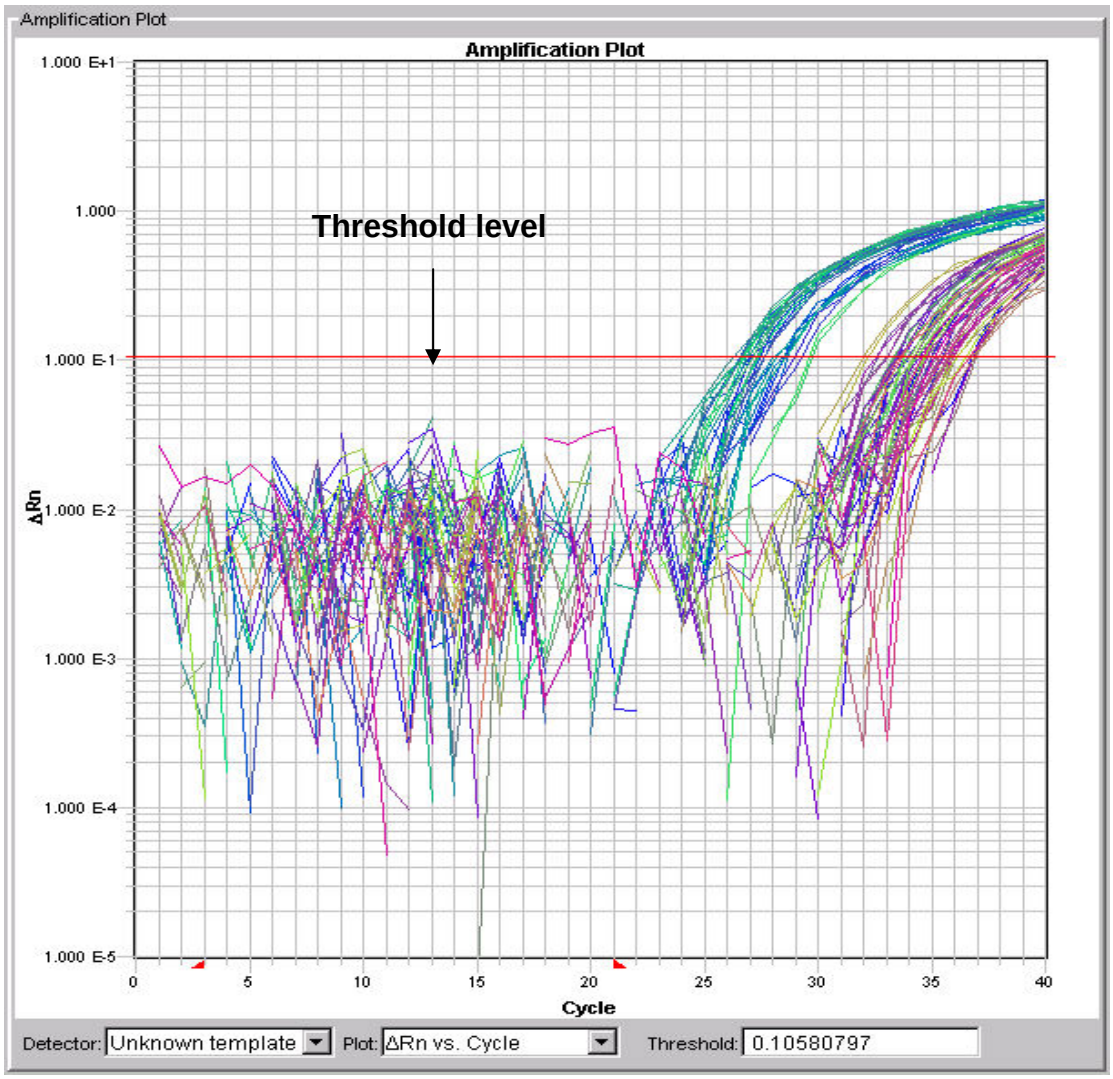

Figure 2.9 Example of an amplification plot after a Taqman RT-PCR reaction.

The Ct value is directly associated to the amount of PCR product and is consequently related to the original amount of target mRNA present in the reaction. A change in Ct value of one, equates to a two-fold difference in initial template concentration, for example, if the Ct value decreases by one compared to the control sample, this is due to a doubling of the initial template concentration. In order to control for mRNA variation between samples, 18S ribosomal RNA was used as an internal control.

The comparative Ct method is used to analyse changes in the expression of a target gene relative to a reference sample, for example a control or RNA from normal tissue. To analyse the results, the ΔCt value was calculated, where ΔCt is the difference between the FAM Ct and the 18S Ct value. This allows the amplified signal to be normalised against the total mRNA content. The mean ΔCt between triplicates was calculated and then used to calculate ΔΔCt, which represents the difference between the ΔCt of a treatment sample compared to the control sample run in that experiment. Experiments run in this thesis were all compared to rat testis total RNA, purchased from Applied Biosystems. The amount of amplified target is given as $2^{-\Delta\Delta Ct}$. This formula is based on the mathematical equation that describes the exponential amplification of the PCR reaction $X_n = X_0 \times (1+E_x)^n$, where X_n is the number of target molecules at the threshold at cycle n, X_0 is the initial number of target molecules, $(1+E_x)$ is the efficiency of the target amplification and n is the number of cycles. If the efficiencies of the target and internal control reactions are equal, the $2^{-\Delta\Delta Ct}$ value provides a measure of relative quantification, showing the fold increase or decrease in mRNA expression in samples, in relation to the control, where each control always has the $2^{-\Delta\Delta Ct}$ value of one.

2.10.6 Validation

To analyse results using the comparative CT method, primers must be validated to ensure that the efficiencies of the target and endogenous control (18S) amplifications are approximately equal. A five-fold dilution series of cDNA reverse-transcribed from rat testis total RNA was run in triplicate. The concentration of RNA was then log transformed and plotted against the

average CT value for that triplicate. A valid primer set should have a slope of -3.3 and a correlation coefficient (R^2 value) of >0.95.

2.11 Statistical analysis

Values are expressed as means ±SEM, and data were analysed using Student's unpaired *t* test or one-way ANOVA followed by the Bonferroni post test, comparing all single and combined treatments with controls and with each other, using GraphPad Prism (version 4, GraphPad software Inc., San Diego, CA). Only where there were statistical significant differences within the multiple comparison analyses were these differences indicated by asterisks. The following criteria were used to determine levels of significant difference:

* P<0.05, ** P<0.01 and *** P<0.001.

2.12 Commonly used solutions

Acid alcohol	70% ethanol 1% concentrated HCl
Bouin's solution	Picric acid can now only be bought by industry so Bouin's solution is made by Triangle Biomedical Sciences Ltd, Lancashire, UK.
Citrate buffer	Citric acid 42.02g (monohydrate; Sigma) Distilled H_2O 1900ml Add concentrated NaOH to pH 5.5 Make up to 2L and pH to 6 Use as 0.01M, diluting in distilled H_2O
Harris's Haematoxylin	2.5g Haematoxylin + 25ml absolute alcohol

	50g aluminium potassium sulphate + 500ml distilled H_2O
	Combine both solutions and boil
	Add 1.25g mercury oxide
	Cool solution in ice then filter
	Add 4ml glacial acetic acid/100ml Haematoxylin
Fast blue buffer	Tris (BDH) 12.1g
	Distilled H_2O 950ml
	Make up to 1L at pH 8.2 with concentrated HCl
	Take 98ml of solution from above, add
	20mg Napthol AS-MX phosphate (Sigma)
	2ml Dimethyl formamide (Sigma)
Scott's Tap water	Potassium chloride 10g
	Magnesium sulphate 100g
	Tap water 5L
TBS	Tris (Sigma) 60.5g
	NaCl (Sigma) 87.6g
	HCl (BDH) 300ml
	Adjust to pH 7.4 using concentrated HCl

3 Identification of cellular targets for androgens in the fetal testis

3.1 Introduction

Testis formation and early development are known to be hormone-independent (Warren et al., 1973), but impaired testosterone production/action may contribute to, or exacerbate, testicular maldevelopment (Sharpe, 2006). For example, patients with complete androgen insensitivity syndrome ('testicular feminization') can exhibit focal dysgenesis in their testes (Hannema et al., 2006), are at increased risk of developing testicular germ cell cancer (Verp and Simpson, 1987), and are also cryptorchid (Sharpe, 2006). This suggests that androgens may play a role in normal testis development subsequent to its formation. This thinking was reinforced by a study in testicular feminized mice (*tfm*) showing that Sertoli cell number was dramatically reduced at birth (Johnston et al., 2004). Since the number of Sertoli cells per testis determines how many germ cells can be supported through spermatogenesis (Sharpe et al., 2003), a reduction in Sertoli cell number during fetal life, is likely to result in a lower sperm count in adulthood. Low sperm counts are also a feature of testicular dysgenesis syndrome (TDS), a hypothesised syndrome that proposes that testicular cancer, cryptorchidism, hypospadias and low sperm counts have a common fetal origin and arise when maldevelopment (dysgenesis) of the fetal testis results in hormonal malfunctions and abnormal development and function of the somatic cells (Sharpe and Skakkebaek, 2003; Skakkebaek et al., 2001). It is thought that the suppressed testosterone levels associated with TDS may account for the low sperm counts, via a reduction in Sertoli cell proliferation/number. These were major motivating factors for the studies in

this thesis. In order to investigate the possibility that androgens play a role in fetal testis development, and in particular in regulating Sertoli cell proliferation, it was necessary to expand and clarify understanding of; 1) the cellular targets for androgen action in the fetal testis; 2) the age at which the androgen receptor (AR) is first expressed, and 3) the levels of testosterone that the fetal testis is exposed to at different gestational stages. These parameters were therefore investigated and related to expansion in testicular cell numbers (Sertoli and peritubular myoid cells). Thus Sertoli cell number was determined at e17.5 and e21.5 in control rats and the change in Sertoli cell number compared with the change in testicular testosterone levels during this period of development. Since it was already known that fetal Sertoli cells do not express the AR (Williams et al., 2001), any effects that androgens may have on fetal Sertoli cells must therefore be mediated via another cell type, most likely to be the AR-positive peritubular myoid cells. Consequently, peritubular myoid cell number was also investigated at e17.5 and e21.5 to examine the possibility that androgens also regulate their proliferation.

3.2 Materials and Methods

In brief, testes were removed from control (vehicle only) fetal rats at e15.5, e17.5, e19.5 and e21.5 (section 2.4), were analysed for AR expression (section 2.6.1), intratesticular testosterone levels (section 2.8.1) and for Sertoli cell and peritubular myoid cell number (sections 2.7.1 and 2.7.2). Sertoli cell number was also determined in day 2 testes from ARKO and PGK-Cre mice (section 2.3). The number of animals used is indicated in each figure legend. In all cases, animals came from a minimum of three litters.

3.2.1 Sertoli cell/peritubular myoid cell ratio

In order to calculate this ratio, the number of Sertoli cells is divided by the number of peritubular myoid cells. However, Sertoli cell and peritubular myoid cell counts were not always performed using testes from the same animals. Consequently, the mean Sertoli cell number was used and divided by each of the individual peritubular myoid cell counts. These calculations were also executed by dividing the individual Sertoli cell counts by the mean peritubular myoid cell number, and the resulting ratios showed little difference to those used in this chapter.

3.3 Results

3.3.1 Sertoli cell number in ARKO mice

Data from Johnston et al, 2004, suggesting that Sertoli cell number per testis is reduced at birth in *tfm* mice was confirmed in mice with complete knockout of the androgen receptor (ARKO mice). On postnatal day (pnd) 2, ARKO mice exhibited a significant 49% reduction in Sertoli cell number, compared to control animals (Figure 3.1). This therefore confirmed the findings by Johnston et al (2004).

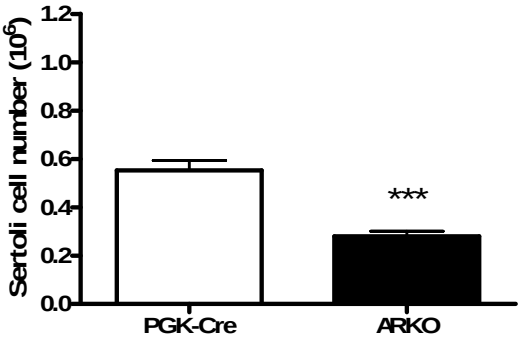

Figure 3.1 Number of Sertoli cells in PGK-Cre (controls; n=5) and ARKO (n=5) day 2 mice testes. Values are means ± SEM. *** $p<0.001$ in comparison to control value.

3.3.2 Androgen receptor expression

Having shown that in the absence of normal androgen action, Sertoli cell number was affected in mice, the expression pattern of AR and onset of its expression were established in rats. Control fetal testis sections from rats aged e15.5, e17.5, e19.5 and e21.5 were immunostained for AR expression and a high magnification review of the sections revealed that AR expression first occurred at e15.5 in some, but not all, of the peritubular myoid cells. By e17.5 all peritubular myoid cells expressed AR, as did a number of interstitial cells and this pattern of expression remained through to e21.5 (Figure 3.2).

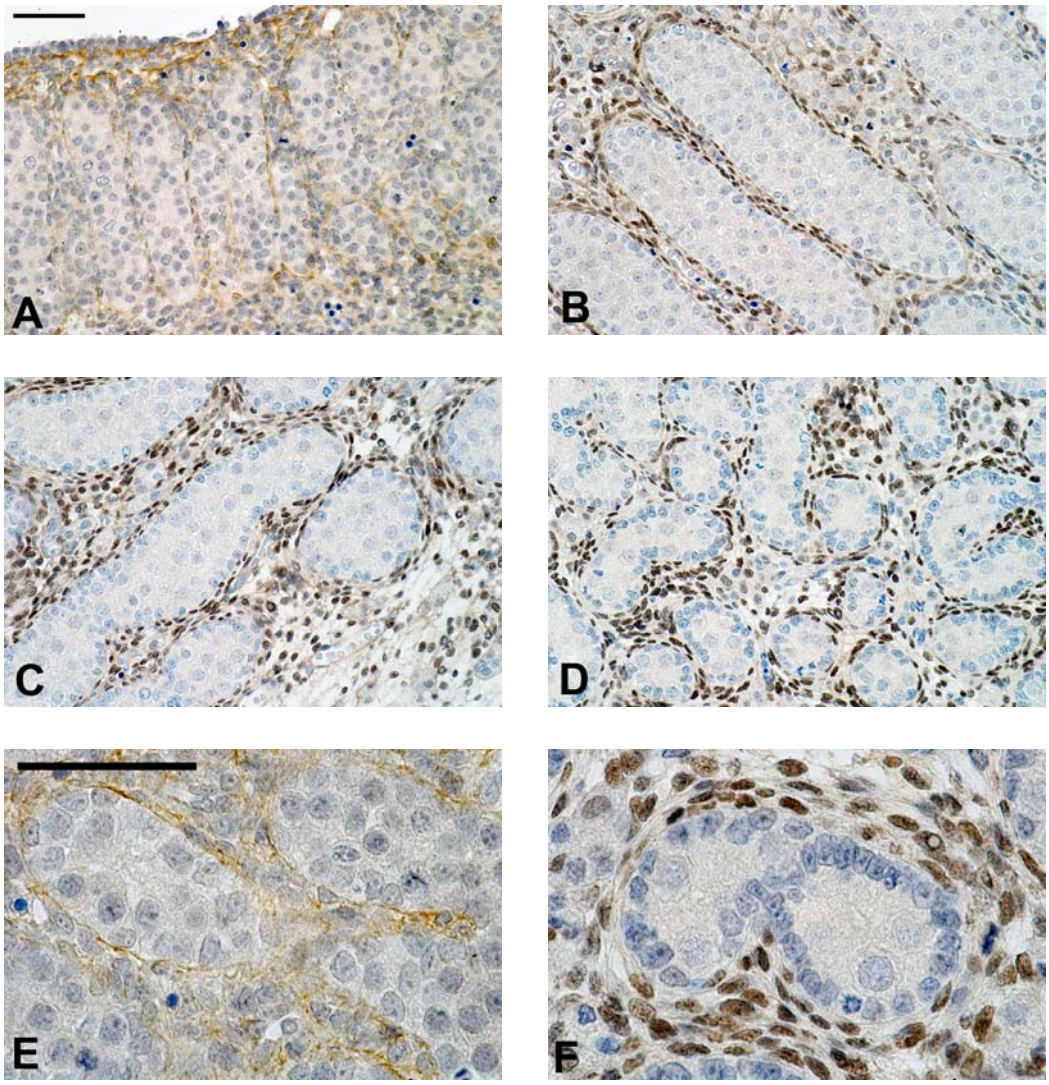

Figure 3.2 AR immunostained sections of e15.5 (A & E), e17.5 (B), e19.5 (C) and e21.5 (D & F) rat testes from control animals. Scale bar represents 50μm.

In order to determine whether the interstitial cells, seen to express AR from e17.5 onwards (Figure 3.2), were fetal Leydig cells, double immunohistochemistry was performed. Sections of e21.5 testis were immunostained for both AR and 3β-HSD, a steroidogenic enzyme present in Leydig cell cytoplasm. Analysis of these sections clearly demonstrated two sub-groups of interstitial cells; the 3β-HSD^{+} AR^{-} fetal Leydig cells and the 3β-HSD^{-} AR^{+} unknown interstitial cells (Figure 3.3). It is also important to note

that as previously described (Williams et al., 2001), the Sertoli cells and germ cells did not express AR during fetal life (Figures 3.2 and 3.3).

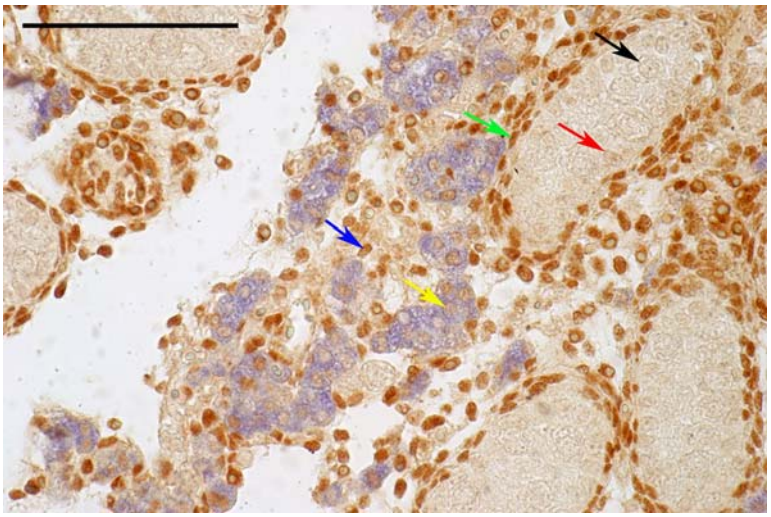

Figure 3.3 A representative photomicrograph depicting double immunostaining of an e21.5 control fetal rat testis section, for AR (Dab = brown) and 3β-HSD (Fast blue = blue). Black arrow depicts AR-negative germ cell, red arrow depicts AR-negative Sertoli cell, green arrow shows AR-positive peritubular myoid cell, yellow arrow shows AR-negative fetal Leydig cell and blue arrow shows unknown AR-positive interstitial cell. Scale bar represents 100μm.

3.3.3 Testicular testosterone levels

Testicular testosterone levels were measured at e15.5, e17.5, e19.5 and e21.5. Levels of testicular testosterone were at their lowest at e15.5 (0.1ng/testis), increased 7 fold to 0.7ng/testis at e17.5, peaked at e19.5 (1.8ng/testis) and then decreased slightly to 1.6ng/testis at e21.5 (Figure 3.4).

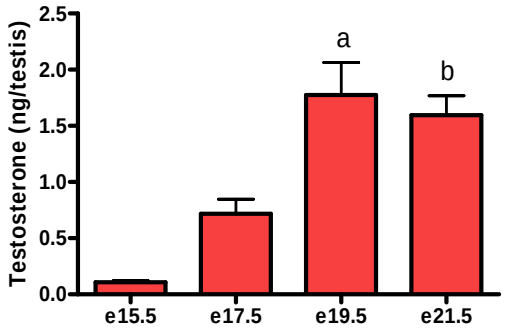

Figure 3.4 Intratesticular testosterone levels in e15.5 (n=4), e17.5 (n=5), e19.5 (n=6) and e21.5 (n=17) control rat testes, expressed as ng/testis. Values are means ± SEM. a: $p<0.01$ compared to e15.5 value, b: $p<0.01$ compared to e15.5 value.

Testicular testosterone levels are typically expressed 'per testis' (Figure 3.4), but in comparing testosterone levels at different ages, when the testes are clearly different with regard to their weight/volume, it seemed more appropriate to express the values 'per testis unit weight'. It was thought that taking into account the weight/volume of the testis would give a more accurate representation of the concentration of testosterone within the testis.

The pattern of testosterone production between e15.5 and e21.5 changed dramatically when expressed 'per testis unit weight'. Testicular testosterone levels increased two fold from e15.5 to peak at 3.6 ng/testis unit weight on e17.5. Testicular testosterone then decreased by 47% to 1.9 ng/testis unit weight on e19.5 and decreased by a further 51% to 0.9 ng/testis unit weight on e21.5 (Figure 3.5).

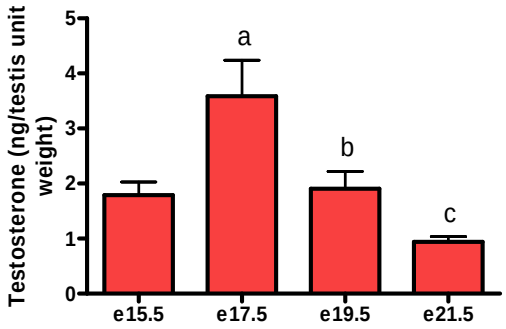

Figure 3.5 Intratesticular testosterone levels in e15.5 (n=4), e17.5 (n=5), e19.5 (n=6) and e21.5 (n=17) control rat testes, expressed as ng/testis unit weight. Values are means ± SEM. a: p<0.01 cmpared to e15.5 value, b: p<0.01 compared to e17.5 value, c: p<0.001 compared to e17.5 value.

3.3.4 Sertoli cell number

In order to further investigate the possibility that androgens play a role in Sertoli cell proliferation, sections of control e17.5 and e21.5 testis were immunostained for WT-1, to enable the visualisation and enumeration of the Sertoli cells during the period when testosterone levels are either increasing or have reached their fetal peak. The number of Sertoli cells per testis increased 7.5 fold from e17.5 to e21.5 (Figure 3.6).

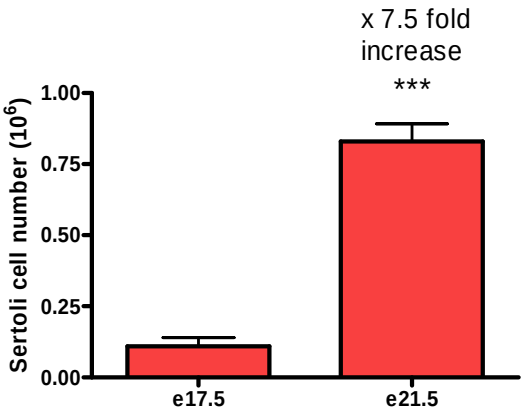

Figure 3.6 Number of Sertoli cells in e17.5 (n=6) and e21.5 (n=19) control fetal rat testes. Values are means ± SEM. *** p<0.001 in comparison to e17.5 value.

3.3.5 Peritubular myoid cell number

Since fetal Sertoli cells do not express AR, any effects that androgens have are likely to be mediated by another cell type, most likely that AR-positive peritubular myoid cell. Consequently, peritubular myoid cell number was also determined at e17.5 and e21.5 to investigate the possibility that androgens also act to regulate their proliferation. The number of peritubular myoid cells per testis increased 11 fold from e17.5 to e21.5 (Figure 3.7).

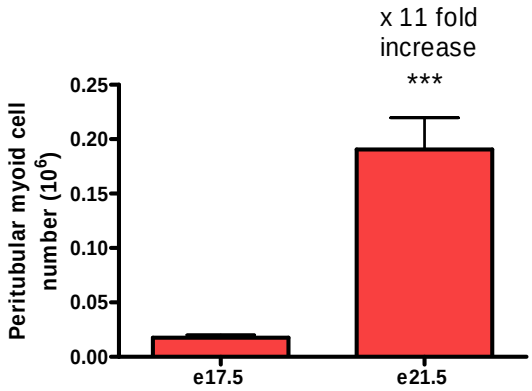

Figure 3.7 Number of peritubular myoid cells in e17.5 (n=5) and e21.5 (n=6) control fetal rat testes. Values are means ± SEM. *** $p<0.001$ in comparison to e17.5 value.

3.3.6 Sertoli cell/peritubular myoid cell ratio

The ratio of Sertoli cells (mean value) to peritubular myoid cells at e17.5 and e21.5 was investigated and revealed that the average number of Sertoli cells per peritubular myoid cell decreased from e17.5 to e21.5, reflecting the greater increase in peritubular myoid cell number (11 fold), compared to the increase seen in Sertoli cell number (7.5 fold) (Figure 3.8).

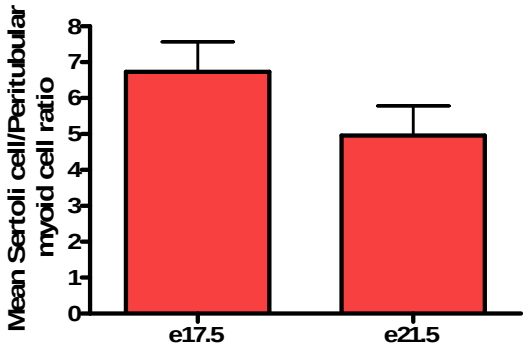

Figure 3.8 Mean Sertoli cell to peritubular myoid cell ratio in e17.5 (n=5) and e21.5 (n=6) control fetal rat testes.

3.4 Discussion

The aim of this chapter was to identify 1) the cellular targets for androgen action, 2) the onset of AR expression and 3) the pattern of testicular testosterone production during the last week of gestation in the fetal rat testis. These investigations were performed to determine whether androgens could play a role in fetal testis development, as suggested by the ARKO data, which demonstrated that in the absence of functioning AR, Sertoli cell number was reduced by 50%.

Immunostaining of testis sections from control fetuses aged e15.5, e17.5, e19.5 and e21.5, revealed that the AR is expressed in some peritubular myoid cells and interstial cells at e15.5, and in all peritubular myoid cells by e17.5, and that this pattern of expression continued through to e21.5. These results are consistent with a previous report that described the location and timing of

AR expression (You and Sar, 1998). However, the present results also demonstrate that the interstitial cells expressing AR during fetal life are not the fetal Leydig cells. The Sertoli cells and germ cells were AR-negative, and this has also been reported previously (Sharpe, 2005; Sharpe et al., 2003; Williams et al., 2001), although there has been some controversy as to whether or not fetal germ cells express AR, with a recent paper reporting the presence of functional AR protein in fetal germ cells (Merlet et al., 2007). This discrepancy is unexplained, but it is established from germ cell transplantation studies that these cells do not require a functional AR to develop into normal fertile sperm (Johnston et al., 2001), whereas a functional AR in the Sertoli cells is essential for normal fertility (Tan et al., 2005).

The pattern of testicular testosterone production during the last week of gestation varied, depending on whether it was expressed as ng/testis or ng/testis unit weight. When expressed as ng/testis, the pattern of testicular testosterone production was almost identical to published data that reported an increase from e15.5, with a peak at e19.5 and then a slight decline at e21.5 (El-Gehani et al., 1998b). However, other publications have suggested that the testosterone peak occurs at e18.5 (Habert and Picon, 1984) or gestational day 20 (Parks et al., 2000). Expressing testicular testosterone levels as ng/testis unit weight changed the pattern of testicular production dramatically, with the fetal testosterone peak occurring at e17.5, instead of e19.5. This peak now coincides with the critical window of masculinisation, reported to occur between e15.5 and e17.5, although this has currently only been shown to affect peripheral androgen targets, such as AGD and Wolffian duct development (Welsh et al., 2007). The present and published data clearly demonstrate that it is very difficult to determine the exact point at

which testicular testosterone levels peak, suggesting that it would be more beneficial to acknowledge that testicular testosterone levels are at their highest around e17.5 to e19.5. The presence of AR expressing cells from e15.5 within the fetal testis, and the high levels of testicular testosterone produced during this period, certainly implies that androgens play a role in cell development in the fetal testis.

Whichever way the testicular testosterone data is interpreted, the high levels of testosterone present in the testis between e17.5 and e21.5 coincide with a significant increase in Sertoli cell number from e17.5 to e21.5. This parallel increase in testicular testosterone and Sertoli cell number also applies to the even greater increase in peritubular myoid cells, from e17.5 to e21.5, suggesting that both Sertoli cell and peritubular myoid cell proliferation rates could be regulated by androgens. This data supports a previous publication that demonstrated high levels of peritubular myoid cell proliferation at gestational day 18 (20%), which declined after birth (Palombi et al., 1992), coincidental with the decline in testosterone.

The data from this chapter, in which Sertoli cell numbers are halved at pnd2 in ARKO mice and Sertoli cell number increases in parallel with testicular testosterone levels in fetal rats, led to the hypothesis that androgens play a role in Sertoli cell proliferation. There have been subsequent publications that also support this hypothesis. Atanassova et al, 2005, described the effects of single and combined treatments, which manipulated FSH, testosterone and oestrogen levels, on Sertoli cell number neonatally in rats. It was concluded that androgens increased Sertoli cell number. Johnston et al, 2004, used testicular feminized (*tfm*) mice, to demonstrate that in the absence of functional AR, Sertoli cell number was reduced by 50% at birth and by 75%

in adulthood. The latter finding suggests that androgens are necessary for normal Sertoli cell proliferation in fetal and postnatal life, with their effects probably being more important in fetal than in postnatal life, when factors such as FSH may become more important (Sharpe et al., 2003).

Tan et al, 2005, also demonstrated that Sertoli cell-selective androgen receptor knockout (SCARKO) mice have Sertoli cell numbers that are comparable to control mice, confirming that AR expression in Sertoli cells is not necessary to obtain normal levels of Sertoli cell proliferation in perinatal life. This latter result, along with evidence that Sertoli cells do not express AR during fetal life (Williams et al., 2001), supports the additional hypothesis that androgens may act through the AR-positive peritubular myoid cells in order to exert an effect on Sertoli cell proliferation in fetal/neonatal life. This interpretation is further supported by evidence that demonstrates that communication exists between the peritubular myoid cells and Sertoli cells, and is essential to testicular development and function. *In vitro* systems using both co-cultures of peritubular myoid cells and Sertoli cells (Skinner and Fritz, 1985a) and isolated Sertoli cells (Buzzard et al., 2003a), have demonstrated that peritubular myoid cell secretions modulate Sertoli cell function, and that activin A produced by peritubular myoid cells may stimulate Sertoli cell proliferation, respectively. Communication is also evident between the Sertoli cells and peritubular myoid cells when they cooperate to produce and lay down the basal lamina (Skinner et al., 1985; Tung, 1984).

This role for peritubular myoid cell to Sertoli cell communication/regulation may also explain why the peritubular myoid cells proliferate at a greater rate than the Sertoli cells, resulting in greater ratio of peritubular myoid cells to

Sertoli cells, as seen in this chapter. Perhaps the peritubular myoid cells need to out-number the Sertoli cells in order to exert an effect on them. Another explanation for the rapid proliferation of the peritubular myoid cells could be due to the role they play in later life, in enabling the transportation of the spermatozoa to the epididymis by inducing peristalsis-like waves and impulses in the seminiferous tubules (Romano et al., 2005).

Since Sertoli cell number in adulthood is the primary determinant of sperm count in humans (Sharpe, 2005; Sharpe et al., 2003), impaired androgen production/action within the fetal testis is one mechanism via which fetal events could lead to low sperm counts in adulthood. Consequently, this hypothesis will be investigated further in chapter 4. The possibility that androgens may act to regulate Sertoli cell proliferation via peritubular myoid cells, and may modulate proliferation of the peritubular myoid cells themselves, will be explored in chapter 7.

Patients with complete androgen insensitivity syndrome (CAIS) are the human equivalent of *tfm* mice. It has been reported that they often exhibit focal areas of testicular dysgenesis (Hannema et al., 2006) and are at considerably increased risk of testicular germ cell cancer (Cools et al., 2005; Verp and Simpson, 1987). *In utero* exposure to DBP has also been reported to cause a TDS-like spectrum of abnormalities (Barlow and Foster, 2003; Ema et al., 1998; Ema et al., 2000; Fisher et al., 2003; Mylchreest et al., 1998; Mylchreest et al., 1999; Mylchreest et al., 2000), including reduced testicular testosterone, multinucleated gonocytes (Fisher et al., 2003; Parks et al., 2000; Shultz et al., 2001) and fetal Leydig cell aggregation (Mahood et al., 2005; Mahood et al., 2006). It is possible that if androgens are regulating Sertoli cells indirectly, they may also have an effect on the germ cells, even though

they do not express AR during fetal life, which could explain the increased risk of germ cell cancer in patients who have CAIS. Dysgenesis and testis cancer has not been reported in ARKO or *tfm* mice, but the possibility that reduced androgen action may also cause testicular dysgenesis will be investigated in chapter 5.

4 Androgens and Sertoli cell proliferation/number

4.1 Introduction

In the previous chapter, it was demonstrated that androgens are likely to play a role in Sertoli cell proliferation, since in the absence of a functioning androgen receptor, as seen in ARKO mice, Sertoli cells numbers are reduced by 50%. The experiments described in this chapter were designed to investigate this hypothesis further, examining the effects of manipulating androgen action, both *in vivo* and *in vitro*, on Sertoli cell number/proliferation.

4.1.1 *In vivo* approach

Pregnant rats were exposed from e13.5 to e20.5, to treatments or co-treatments designed to manipulate testosterone levels (DBP, testosterone propionate; TP) or action (flutamide, DMBA), and the effects that these treatments had on Sertoli cell proliferation/number were analysed at e21.5. Further details on why these treatments were chosen and administered at the doses described below (Table 4.1) can be found in section 2.2.

Since *in utero* exposure to DBP 500mg/kg had the most profound effect on intratesticular testosterone levels and Sertoli cell number, exposure to this chemical was investigated further. In addition to administering DBP at 500mg/kg, from e13.5-20.5, other pregnant dams were treated with 100mg/kg, 20mg/kg or 4mg/kg, to evaluate whether testicular testosterone and Sertoli cell number responded to DBP in a dose dependent manner. DBP 500mg/kg was also administered during different gestational time windows; from e13.5-15.5 (early), e15.5-17.5 (middle), e19.5-20.5 (late), e13.5-20.5 (standard) or e11.5-20.5 (extended) and intratesticular testosterone, AGD and

Sertoli cell numbers analysed at e21.5. These time windows were investigated to ascertain whether androgens act to modulate Sertoli cell proliferation during specific time windows, as seen in the development of the Wolffian duct (Welsh et al., 2007), or whether they act throughout the later stages of gestation, once the seminiferous cords are formed (standard window) or even before this (extended window). Exposure to DBP during the middle and late windows permitted evaluation of the relative importance of testosterone and FSH which is known to play a key role in Sertoli cell proliferation (Orth, 1984) and is first detectable at e19.5 (Majdic et al., 1997). Intratesticular testosterone, Sertoli cell number and proliferation indices were also investigated at time points during the middle (e17.5) and late (e21.5) treatment windows, in control and DBP-exposed animals, to establish whether DBP reduced testicular testosterone, Sertoli cell number and proliferation at both ages and whether the levels of reduction were comparable.

4.1.2 *In vitro* approach

An *in vitro* approach was also utilized to examine the effects of manipulating androgen action on Sertoli cell proliferation. Testis explants from e19.5 untreated rats were cultured in the presence of a variety of agents, all expected to influence Sertoli cell proliferation. Testis explants were cultured with hydroxyflutamide, an anti-androgenic compound that competitively blocks the action of both testosterone and dihydrotestosterone (DHT) at the level of the androgen receptor (section 2.2.2), and consequently was expected to reduce Sertoli cell proliferation. In order to test the culture system, explants were also cultured with factors that might be expected to stimulate Sertoli cell proliferation; FSH has been shown to stimulate Sertoli cell

proliferation *in vivo* (Allan et al., 2004; Johnston et al., 2004; Meachem et al., 1996); hCG is structurally similar to LH and as such has been shown to stimulate increased testosterone production in testis explants (Hallmark et al., 2007); and EGF has been shown to stimulate Sertoli cell proliferation *in vitro*, postnatally (Petersen et al., 2001). Cyclopamine was also administered as it inhibits the Desert hedgehog (Dhh) pathway (King, 2002) between Sertoli cells (Bitgood et al., 1996,) and peritubular myoid cells and Leydig cells, as they express its receptor Patched 1 (PTCH) (Clark et al., 2000). Previous data has shown that when the Dhh pathway is inhibited, cord formation is disrupted (Yao and Capel, 2002) and Leydig cells do not undergo complete differentiation (Yao et al., 2002). In mice lacking Dhh, 92.5% of the males were under-masculinised, with abnormal peritubular myoid cells and severely restricted spermatogenesis (Clark et al., 2000). Consequently, testis explants were exposed to cyclopamine to investigate the possible effects of disrupting hedgehog signalling pathways between the Sertoli cells and peritubular myoid cells, and incomplete Leydig cell differentiation, on Sertoli cell proliferation.

4.2 Materials and Methods

4.2.1 *In vivo* methods

In brief, time-mated pregnant dams were treated daily using one of the regimes shown in Table 4.1.

Table 4.1 Summary of *in utero* treatment regimes used.

Treatment	Treatment window	Kill age
Control (vehicle only)	e13.5-20.5	e21.5
Flutamide 100mg/kg	e13.5-20.5	e21.5
DMBA 400µg/kg	e13.5-20.5	e21.5
TP 20mg/kg	e13.5-20.5	e21.5
DBP 500mg/kg	e13.5-20.5	e21.5
DBP 500mg/kg + Flutamide 100mg/kg	e13.5-20.5	e21.5
DBP 500mg/kg + DMBA 400µg/kg	e13.5-20.5	e21.5
DBP 500mg/kg + TP 20mg/kg	e13.5-20.5	e21.5
DBP 100mg/kg	e13.5-20.5	e21.5
DBP 20mg/kg	e13.5-20.5	e21.5
DBP 4mg/kg	e13.5-20.5	e21.5
DBP 500mg/kg	e13.5-15.5	e21.5
DBP 500mg/kg	e15.5-17.5	e21.5
DBP 500mg/kg	e19.5-20.5	e21.5
DBP 500mg/kg	e13.5-20.5	e21.5
DBP 500mg/kg	e11.5-20.5	e21.5

The pregnant dams were killed at e21.5 and pups removed (section 2.4). Bodyweight (section 2.4.2) and anogenital distance (section 2.4.3) were recorded before testes were removed (section 2.4.1) and weighed. After fixation (section 2.4.6), processing (section 2.4.7) and sectioning (section 2.6.1.1), testes were stained for WT1 expression (section 2.6.1), and Sertoli cell number determined (sections 2.7.1). Frozen testes were assayed for intratesticular testosterone levels (section 2.8.1). The number of animals used is indicated in each figure legend. In all cases, animals came from a minimum of three litters.

4.2.2 *In vitro* methods

Briefly, testes were removed from e19.5 fetuses, from untreated dams (section 2.4), and carefully dissected into explants (section 2.5.1). They were then cultured for 48 hours (sections 2.5.2 and 2.5.3) with a variety of chemicals expected to modulate Sertoli cell proliferation. These treatments are summarised in table 4.2.

Table 4.2 Summary of chemical treatments added to *in vitro* fetal testis explants.

Chemical Treatment	Target Concentration
Hydroxyflutamide	10^{-4}M, 10^{-5}M, 10^{-6}M
FSH (follicle stimulating hormone)	0.05 iu/ml
hCG (human chorionic gonadotrophin)	0.1 iu/ml
FSH + hCG	0.05 iu/ml + 0.1 iu/ml
EGF (epidermal growth factor)	10 nM
Cyclopamine	25 μM

Two hours prior to the end of the culture period the treatment medium was replaced with medium containing BrdU (section 2.2.6), to enable the subsequent identification of proliferating cells. Once the explants were fixed (section 2.4.6), processed (section 2.4.7) and sectioned (section 2.6.1.1), they were immunostained for BrdU (section 2.6.1) and the Sertoli cell proliferation index calculated for each treatment group (section 2.7.3).

4.2.3 Statistical analysis

Data were analysed using one-way ANOVA followed by the Bonferroni post test, except when comparing intratesticular testosterone levels and Sertoli cell proliferation and number in animals killed at e17.5 and e21.5 after exposure to DBP from e13.5. These data were analysed using Student's unpaired *t* test.

4.3 Results

4.3.1 *In vivo* studies

4.3.1.1 Treatments expected to manipulate testosterone levels/action

Fetal bodyweight was not affected by treatment with DBP, flutamide or DMBA alone but was reduced significantly, and to a variable degree, by treatment with DBP + flutamide, DBP + DMBA, DBP + TP and TP alone. Treatment with TP ± DBP caused the most marked decrease (25-34%) in bodyweight (Table 4.3).

Table 4.3 Bodyweights of fetuses at e21.5 in all treatment groups. Means ± SEM.

Treatment	Bodyweight (g)
Control	4.75 ± 0.10 (n=31)
Flutamide	4.76 ± 0.21 (n=7)
DMBA	5.01 ± 0.07 (n=18)
TP	3.55 ± 0.10 (n=29) ***
DBP	4.96 ± 0.06 (n=19)
DBP + Flutamide	3.86 ± 0.11 (n=13) ***
DBP + DMBA	4.34 ± 0.07 (n=20) *
DBP + TP	3.12 ± 0.07 (n=22) ***

*p<0.05, ***p<0.001 in comparison to control value.

Exposure to DBP resulted in a 27% decrease in testis weight compared to control animals, and in males exposed to DBP + TP, testis weight was decreased by a further 14% (Figure 4.1). Flutamide exposure on its own did not induce a reduction in testis weight, but when combined with DBP treatment, resulted in a 37% reduction in testis weight, which was greater than the reduction in animals exposed to DBP alone (Figure 4.1). DMBA exposure did not cause a significant reduction in testis weight but, when combined with DBP resulted in a 23% decrease (Figure 4.1). There was no consistent relationship between treatment-induced reduction in bodyweight and testis weight or between bodyweight and Sertoli cell number (see below).

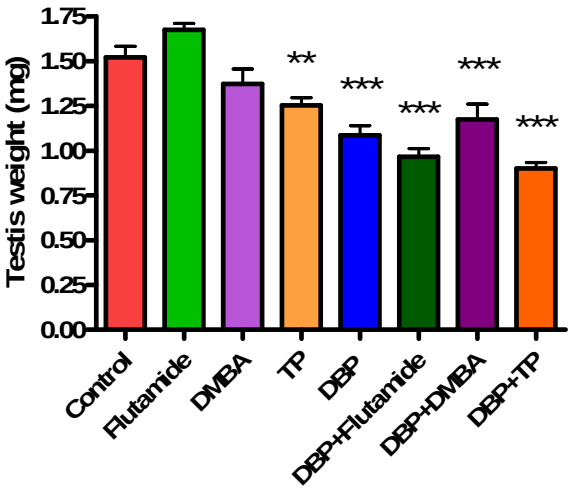

Figure 4.1 Effect of *in utero* exposure to flutamide (n=16), DMBA (n=19), TP (n=28), DBP (n=28) or DBP + flutamide (n=18), DBP + DMBA (n=20), DBP + TP (n=28) or vehicle alone (control; n=28) on testis weight in e21.5 rats. Values are means ± SEM. **p<0.01, ***p<0.001 in comparison to control value.

4.3.1.1.1 Testicular testosterone levels

Testicular testosterone levels (per testis) were significantly reduced in animals exposed to DBP (48% reduction), DBP + flutamide (48%), DBP + DMBA (69%), and DBP + TP (89%), compared to controls. Exposure to either DMBA or TP alone caused a 42% reduction in testicular testosterone, but this was not statistically significant due to high between-animal variation (Figure 4.2).

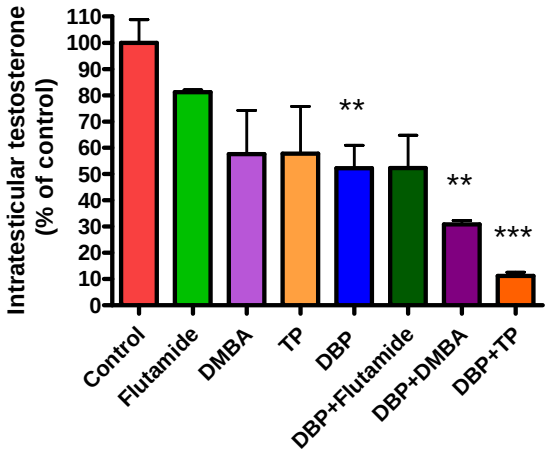

Figure 4.2 Intratesticular testosterone levels at e21.5 in male rats exposed *in utero* to flutamide (n=4), DMBA (n=5), TP (n=5), DBP (n=14) or DBP + flutamide (n=6), DBP + DMBA (n=5), DBP + TP (n=5) or vehicle alone (control; n=11). Testicular extracts were not all assayed together. To avoid consequent inter-assay errors, testosterone levels for testes from treated animals were expressed as a percentage of the mean value for control testes run in the same assay. **p<0.01, ***p<0.001 in comparison to control value.

4.3.1.1.2 Anogenital distance (AGD)

In male pups exposed to flutamide alone or flutamide + DBP, AGD was significantly decreased such that values were similar to those found in female control animals (Figure 4.3). Exposure to DBP alone caused an average 11% reduction in AGD, but this did not reach statistical significance and similar results were found for DBP + DMBA exposed males (Figure 4.3). Exposure to TP alone increased AGD by 22% on average, and when administered with DBP, reversed the reduction in AGD caused by DBP, so there was an overall increase of 14% in AGD when compared to the average control male.

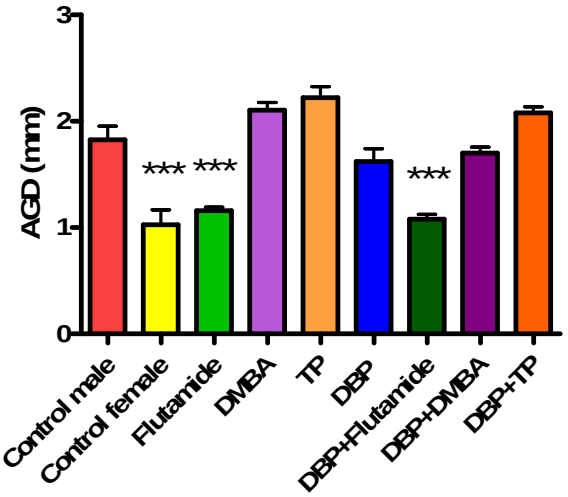

Figure 4.3 Anogenital distance at e21.5 in male rats exposed *in utero* to flutamide (n=12), DMBA (n=19), TP (n=17), DBP (n=11) or DBP + flutamide (n=18), DBP + DMBA (n=20), DBP + TP (n=27) or vehicle alone (control; male n=28 and female n=8). Values are means ± SEM. ***p<0.001 in comparison to control male value.

4.3.1.1.3 Sertoli cell number

Sertoli cell number per testis was reduced by 49% in animals exposed to DBP alone (Figure 4.4). Animals exposed to TP alone also exhibited a significant reduction (33%) in Sertoli cell number, and when DBP + TP treatments were combined, a 59% reduction in Sertoli cell number was evident. Exposure to flutamide alone had no significant effect on Sertoli cell number, and animals exposed to both DBP + flutamide exhibited a similar reduction in Sertoli cell number (56%) to animals treated with DBP alone (49% reduction; Figure 4.4). Exposure to DMBA alone had no significant effect on Sertoli cell number and combined treatment with DBP + DMBA did not result in any greater reduction in Sertoli cell number than after treatment with DBP alone (Figure 4.4).

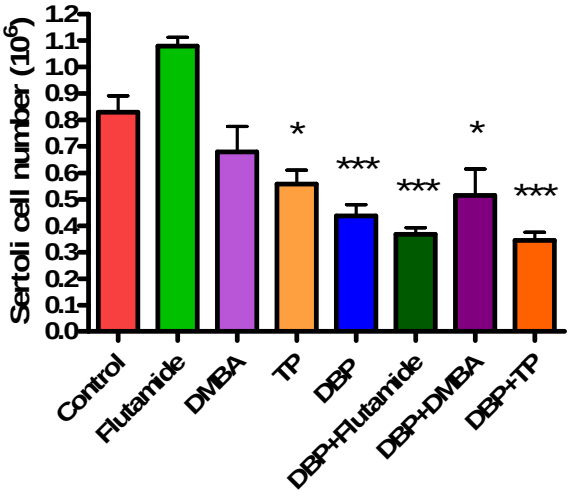

Figure 4.4 Effect of *in utero* exposure to flutamide (n=6), DMBA (n=6), TP (n=8), DBP (n=9) or DBP + flutamide (n=6), DBP + DMBA (n=6), DBP + TP (n=8) or vehicle alone (control; n=19) on Sertoli cell numbers in e21.5 rat testes. Values are means ± SEM. *p<0.05, ***p<0.001 in comparison to control value.

The potential relationship/correlation between the intratesticular levels of testosterone and Sertoli cell number, was evaluated by plotting the mean intratesticular testosterone values (expressed as a percentage of intra-assay control) versus Sertoli cell number. As the latter were not measured in the same animals as those in which the testosterone levels were evaluated, only group mean values were used for analysis. There was a clear positive correlation (p=0.019) between intratesticular testosterone levels and Sertoli cell number (Figure 4.5).

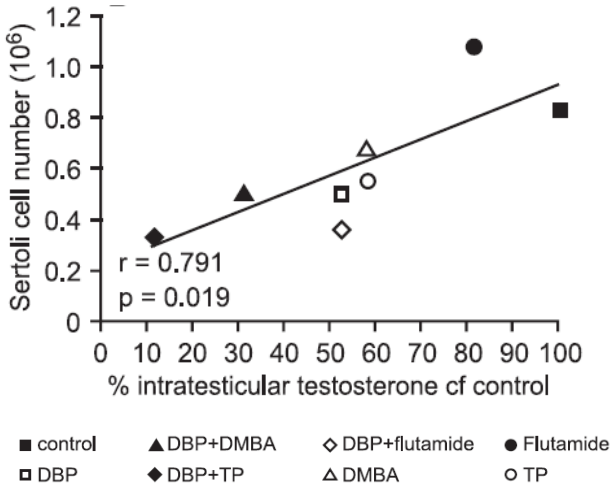

Figure 4.5 Correlations on e21.5 between intratesticular testosterone levels and Sertoli cell number. Each data point represents the mean value for each treatment group. Intratesticular testosterone levels have been expressed as the percentage of the mean control value in the same testosterone assay.

4.3.1.2 DBP dose response

Exposure to DBP 500mg/kg resulted in a 21% decrease in testis weight compared to control animals (Figure 4.6), but exposure to lower doses of DBP (100, 20 or 4mg/kg) had no significant effect on testis weight.

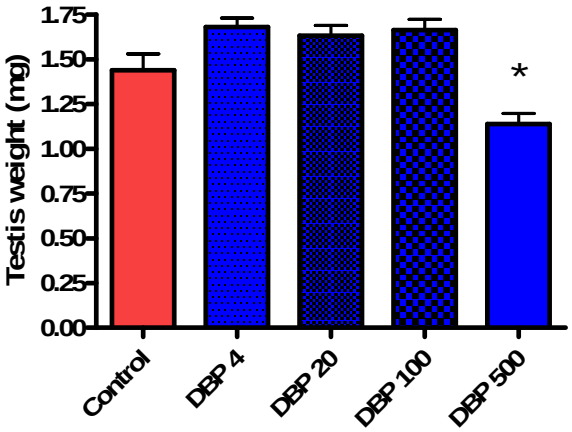

Figure 4.6 Effect of *in utero* exposure to DBP at 4mg/kg (n=31), 20mg/kg (n=30), 100mg/kg (n=27), 500mg/kg (n=31) or vehicle alone (control; n=29) on testis weight in e21.5 rat testes. Values are means ± SEM. *p<0.05 in comparison to control value.

Testicular testosterone levels were significantly reduced in animals exposed to DBP 500mg/kg (84% reduction) and DBP 100mg/kg (59%), compared to controls. Exposure to DBP 20mg/kg and DBP 4mg/kg did not cause a significant change in testicular testosterone levels (Figure 4.7).

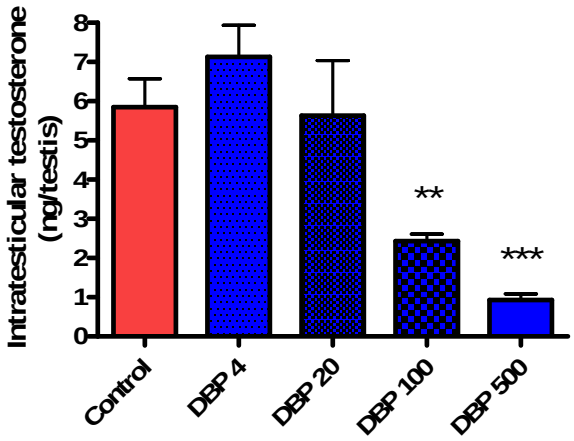

Figure 4.7 Intratesticular testosterone levels in e21.5 rat testes after *in utero* exposure to DBP at 4mg/kg (n=4), 20mg/kg (n=4), 100mg/kg (n=4), 500mg/kg (n=14) or with vehicle alone (control; n=6). Testosterone levels expressed as ng/testis. Values are means ± SEM. **p<0.01, ***p<0.001 in comparison to control value.

Sertoli cell number per testis was reduced by 56% in animals exposed to DBP 500mg/kg (Figure 4.8). Exposure to lower doses of DBP (100, 20 or 4mg/kg) had no significant effect on Sertoli cell number (Figure 4.8).

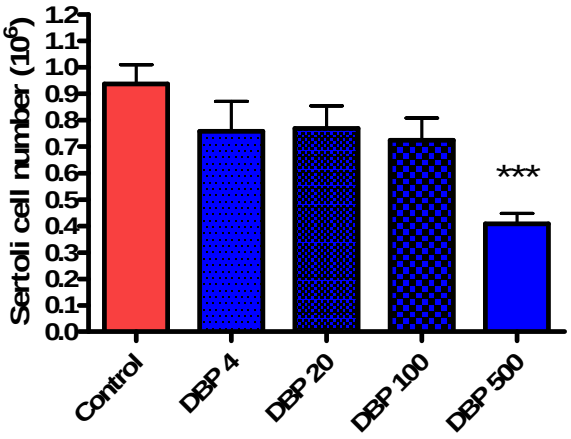

Figure 4.8 Sertoli cell number in e21.5 rat testes after *in utero* exposure to DBP at 4mg/kg (n=6), 20mg/kg (n=9), 100mg/kg (n=9), 500mg/kg (n=9) or with vehicle alone (control; n=12). Values are means ± SEM. ***p<0.001 in comparison to control value.

4.3.1.3 DBP treatment windows

Testis weight was significantly reduced in animals exposed to DBP 500mg/kg during the e13.5-20.5 standard treatment window (35% decrease) and during the e11.5-20.5 treatment window (13% reduction), compared to control animals (Figure 4.9). Exposure to DBP 500mg/kg during the e13.5-15.5, e15.5-17.5 and e19.5-20.5 treatments windows had no significant effect on testis weight.

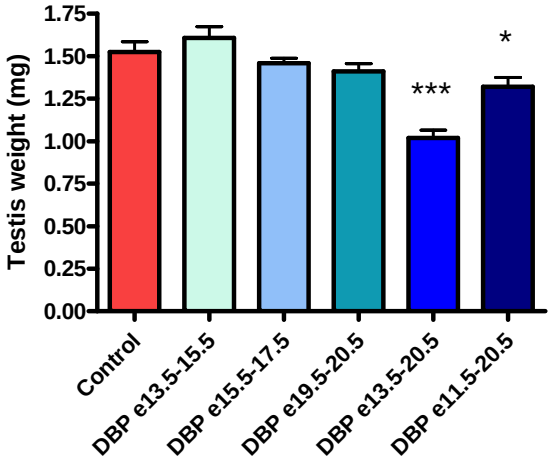

Figure 4.9 Effect of *in utero* exposure to DBP 500mg/kg during the following treatment windows; e13.5-15.5 (n=14), e15.5-17.5 (n=24), e19.5-20.5 (n=27), e13.5-20.5 (n=26), e11.5-20.5 (n=25) or with vehicle alone during the standard e13.5-20.5 treatment window (control; n=29) on testis weight in e21.5 rat testes. Values are means ± SEM. *p<0.05, ***p<0.001 in comparison to control value.

In utero exposure to DBP 500mg/kg during the e13.5-20.5 and e11.5-20.5 treatment windows caused a significant reduction in testicular testosterone levels (72% and 77%, respectively). Exposure during the e19.5-20.5 treatment window caused a similar reduction (70%), although this was only approaching statistical significance, most likely due to a lower 'n' number. Exposure to DBP during the e13.5-15.5 and e15.5-17.5 treatment windows caused a small (30% and 36% respectively) and non-significant reduction in testicular testosterone (Figure 4.10).

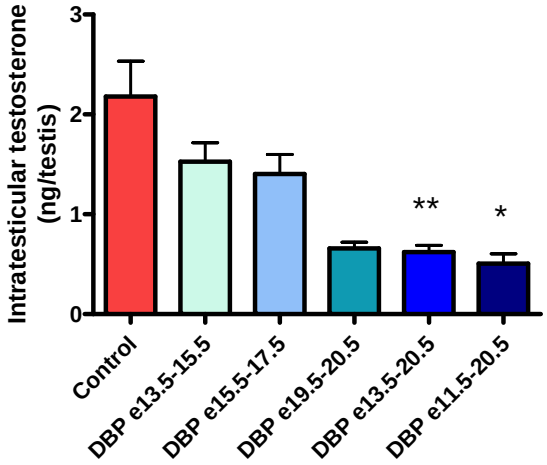

Figure 4.10 Intratesticular testosterone levels in e21.5 rat testes after *in utero* exposure to DBP 500 mg/kg during the following treatment windows; e13.5-15.5 (n=4), e15.5-17.5 (n=6), e19.5-20.5 (n=3), e13.5-20.5 (n=12), e11.5-20.5 (n=5) or with vehicle alone during the standard e13.5-20.5 treatment window (control; n=20). Testosterone levels expressed as ng/testis. Values are means ± SEM. *p<0.05, **p<0.01 in comparison to control value.

Male pups exposed to DBP 500mg/kg during the e15.5-17.5, e13.5-20.5 and e11.5-20.5 treatment windows all showed similar and significant reductions in AGD, however, none of these treatment groups reduced AGD to values comparable with the female controls (Figure 4.11). AGD was not altered in animals exposed to DBP 500mg/kg from e13.5-15.5 (Figure 4.11), and measurements were not taken for animals treated in the e19.5-20.5 window.

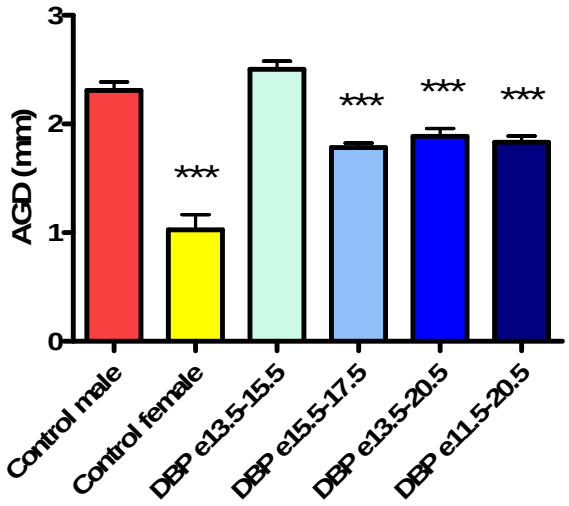

Figure 4.11 Anogenital distance at e21.5 in male rats after *in utero* exposure to DBP 500 mg/kg during the following treatment windows; e13.5-15.5 (n=13), e15.5-17.5 (n=23), e13.5-20.5 (n=20), e11.5-20.5 (n=25) or with vehicle alone during the standard e13.5-20.5 treatment window (control; male n=15 and female n=8). Values are means ± SEM. ***p<0.001 in comparison to control value.

Sertoli cell number per testis was significantly reduced in the e19.5-20.5 (40% reduction), e13.5-20.5 (49% reduction) and e11.5-20.5 (65% reduction) treatment windows, compared to control animals (Figure 4.12). Exposure to DBP 500mg/kg from e13.5-15.5 or e15.5-17.5 had no effect on Sertoli cell number per testis determined at e21.5 (Figure 4.12).

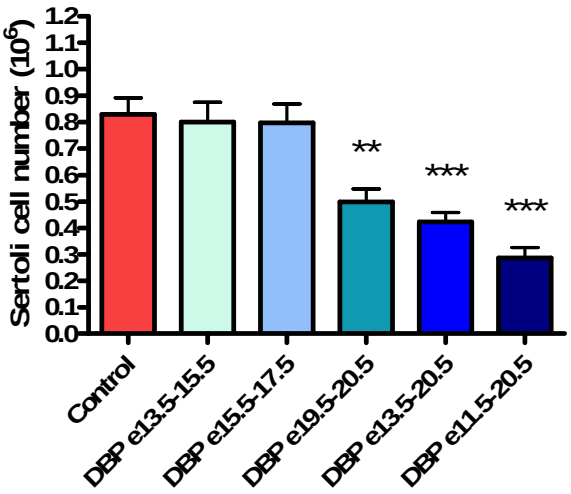

Figure 4.12 Effect of *in utero* exposure to DBP 500 mg/kg during the following treatment windows; e13.5-15.5 (n=5), e15.5-17.5 (n=6), e19.5-20.5 (n=8), e13.5-20.5 (n=11), e11.5-20.5 (n=5) or with vehicle alone during the standard e13.5-20.5 treatment window (control; n=19) on Sertoli cell numbers in e21.5 rat testes. Values are means ± SEM. **p<0.01, ***p<0.001 in comparison to control value.

4.3.1.4 DBP effects on testes from animals exposed from e13.5 and removed at e17.5

In the previous section of this chapter (4.3.1.3), the effects of exposure to DBP during different treatment windows were only examined at e21.5. Consequently any 'early' treatment window effects were potentially masked, since it is possible that the testis may have subsequently recovered from any 'early' DBP treatment effects. Therefore an additional study was undertaken, whereby rats were exposed to DBP from e13.5 and killed at e17.5 or e21.5 to determine the effects of DBP exposure at e17.5 and to compare them with those identified at e21.5.

As well as reducing intratesticular testosterone levels at e21.5, levels were also reduced significantly at e17.5 after exposure to DBP 500mg/kg *in utero* from e13.5 (Figure 4.13); the magnitude of the DBP-induced reduction (53-61%) was similar at both ages.

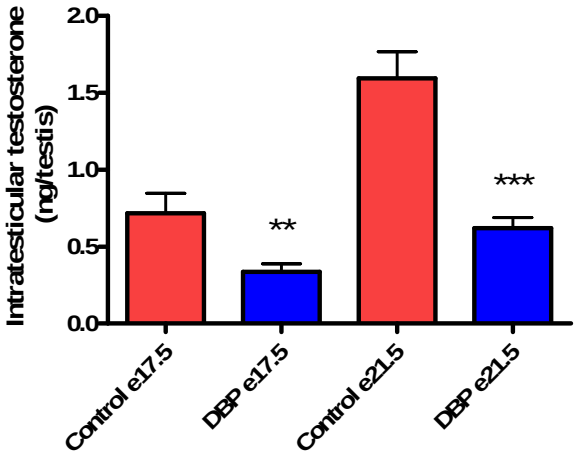

Figure 4.13 Intratesticular testosterone values from control (e17.5 n=5 and e21.5 n=17) and DBP (e17.5 n=8 and e21.5 n=12) animals, treated from e13.5 to the day prior to kill. Values are means ± SEM. **p<0.01, ***p<0.001 in comparison to respective control value.

Sertoli cell number was reduced by 52% in animals exposed to DBP 500mg/kg from e13.5 and killed on e17.5. This reduction was not statistically significant, however, due to high between-animal variation at this age. Animals exposed to DBP from e13.5 until e20.5, and then killed at e21.5 demonstrated a 49%, significant, reduction in Sertoli cell number (Figure 4.14).

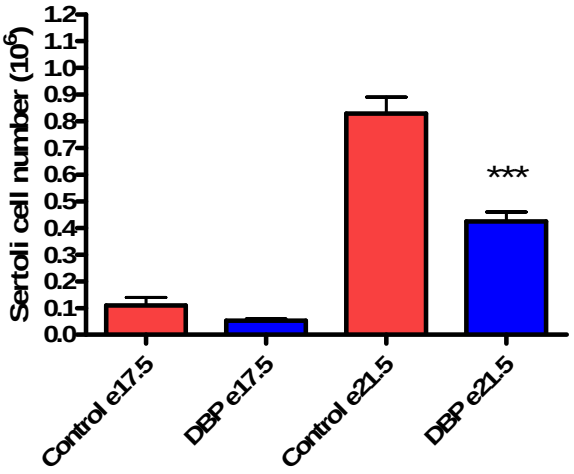

Figure 4.14 Sertoli cell numbers from control (e17.5 n=6 and e21.5 n=19) and DBP (e17.5 n=5 and e21.5 n=11) animals, treated from e13.5 to the day prior to kill. Values are means ± SEM. ***p<0.001 in comparison to respective control value.

Exposure to DBP 500mg/kg did not have a significant effect on the Sertoli cell proliferation index at e17.5 in animals exposed from e13.5 (Figure 4.15). In contrast, animals exposed from e13.5 until e20.5, and then killed at e21.5, did demonstrate a significant reduction in the Sertoli cell proliferation index measured at e21.5 (Figure 4.15).

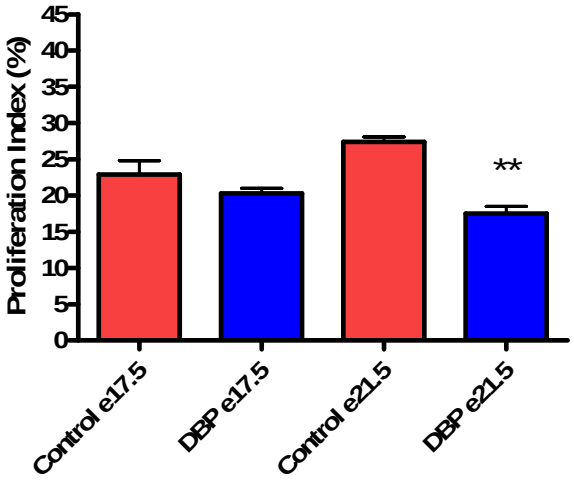

Figure 4.15 Sertoli cell proliferation indices from control (e17.5 n=6 and e21.5 n=4) and DBP (e17.5 n=6 and e21.5 n=5) animals, treated from e13.5 to the day prior to kill. Values are means ± SEM. **p<0.01 in comparison to respective control value.

4.3.2 *In vitro* studies

Testis explants from e19.5 untreated rats were cultured for 48 hours with a variety of chemicals expected to modulate Sertoli cell proliferation rates. Two hours prior to the end of the 48 hour culture period, the culture medium was replaced with medium containing BrdU so that the proliferation index of Sertoli cells could be subsequently analysed. Once testis explants had been fixed, processed and sectioned, they were immunostained for BrdU expression. The photomicrographs below demonstrate that after 48 hours of culture the gross architecture of the testis remained intact (Figure 4.16, A and B) and many of the Sertoli cells, as well as some interstial cells, were proliferating (Figure 4.16, C and D).

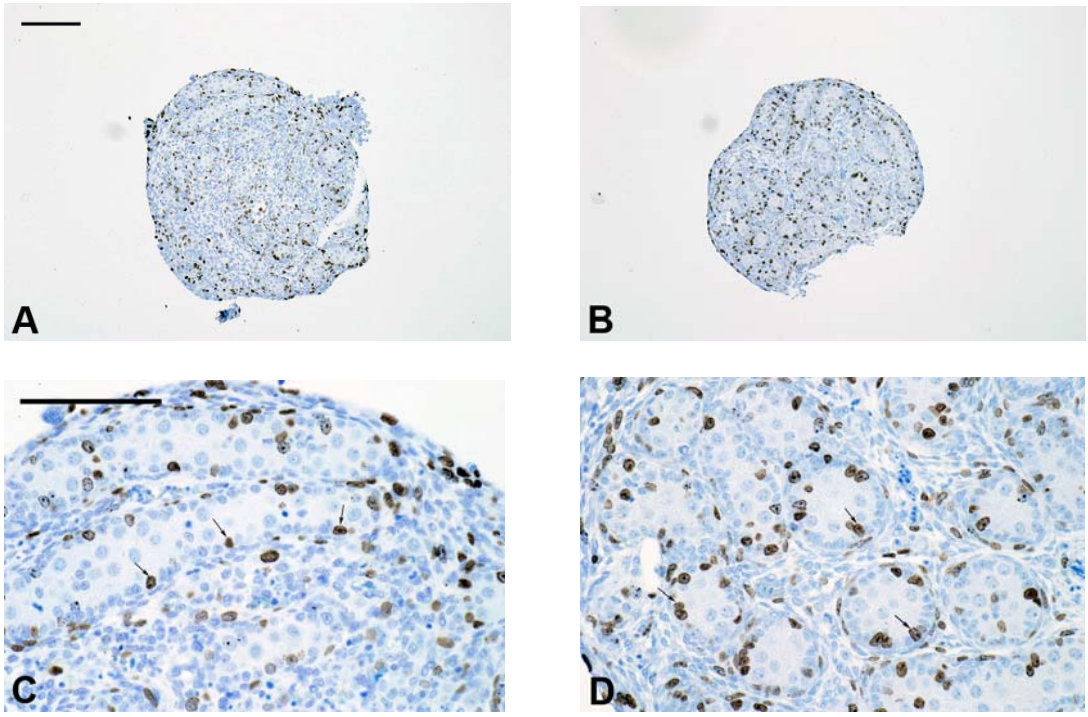

Figure 4.16 Immunoexpression of BrdU in fetal testis explants cultured for 48 hours with either hydroxyflutamide (B and D) or vehicle alone (A and C). Scale bars represent 100μm. Arrows denote proliferating Sertoli cells.

In vitro exposure to hydroxyflutamide, at 10^{-4}M, 10^{-5}M or 10^{-6}M, for 48 hours, had no significant effect on the Sertoli cell proliferation index, when compared to testis explants cultured with vehicle alone (Figure 4.17).

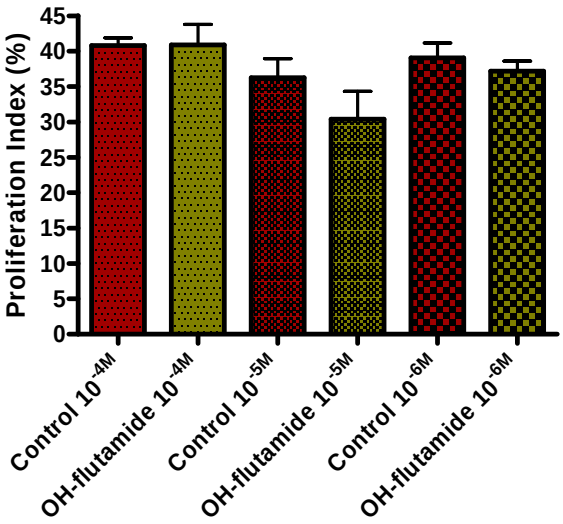

Figure 4.17 Sertoli cell proliferation index of testis explants cultured for 48 hours with hydroxyflutamide (OH-flutamide) at 10^{-4}M (n=4 for OH-flutamide and control), 10^{-5}M (n=3 OH-flutamide and control), and 10^{-6}M (n=4 for OH-flutamide and control).

Culturing testis explants with FSH, hCG, FSH + hCG and EGF also had no significant effect on the Sertoli cell proliferation index when compared to control explants that were cultured with vehicle alone (Figure 4.18).

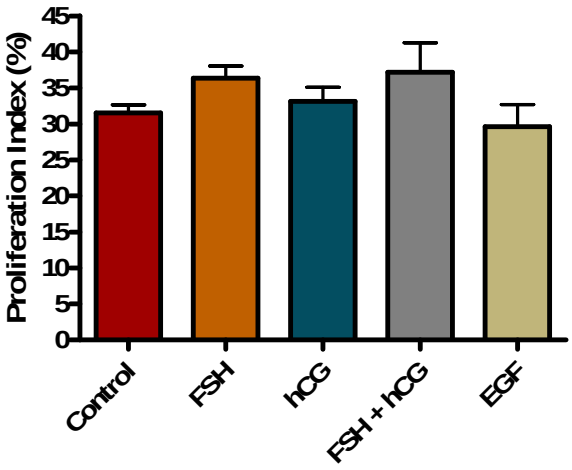

Figure 4.18 Effect of culturing testis explants for 48 hours with FSH (n=6), hCG (n=6), FSH + hCG (n=6), EGF (n=4) or vehicle alone (n=14) on Sertoli cell proliferation index.

Testis explants cultured for 48 hours in the presence of cyclopamine, an inhibitor of the hedgehog signalling pathway, demonstrated a significant reduction in testosterone production, determine by measuring testosterone levels in culture media (Figure 4.19).

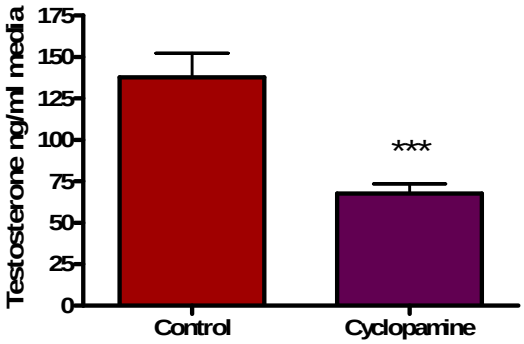

Figure 4.19 Effect of culturing testis explants for 48 hours with cyclopamine (n=8) or vehicle alone (n=8) on testosterone production. Values are means ± SEM. ***$p<0.001$ in comparison to control value.

Testis explants cultured with cyclopamine also exhibited a 20% significant reduction in Sertoli cell proliferation index, compared to explants cultured with vehicle alone (Figure 4.20).

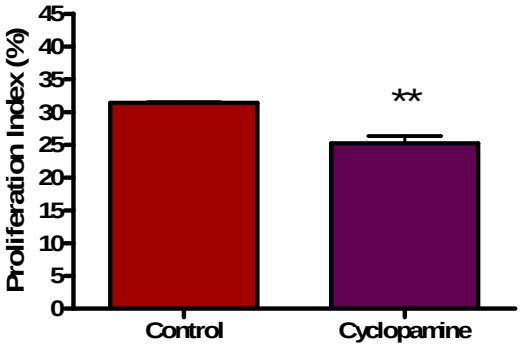

Figure 4.20 Effect of culturing testis explants for 48 hours with cyclopamine (n=4) or vehicle alone (n=4) on the Sertoli cell proliferation index. Values are means ± SEM. **p<0.01 in comparison to control value.

4.4 Discussion

4.4.1 *In vivo* studies

4.4.1.1 Treatments expected to manipulate testosterone levels/action

This study sought to establish whether or not altered androgen levels/action within the fetal testis contributed causally to the reduction in Sertoli cell proliferation/numbers as evidence from ARKO and *tfm* mice have both shown a significant reduction in Sertoli cell number or volume per testis, in the absence of a functioning AR (Johnston et al., 2004; Tan et al., 2005), chapter 3). In order to investigate this, pregnant dams were exposed to a variety of treatments and co-treatments, designed to manipulate testosterone levels (DBP, TP) or action (flutamide and DMBA) in the testis during fetal development. The present data demonstrates that exposure to DBP alone, or after co-treatment with flutamide, DMBA or TP caused a reduction in both Sertoli cell number and intratesticular testosterone levels and, unexpectedly, that the co-administration of DBP + TP resulted in the most severe reduction

in both of these parameters; TP treatment alone also reduced both parameters to some extent. It was also surprising that flutamide exposure had no significant effect on either of the parameters in spite of completely preventing masculinisation of AGD. DMBA had no significant effect on either Sertoli cell number or intratesticular testosterone levels. At the treatment group level, the results clearly demonstrate a positive correlation between intratesticular testosterone and Sertoli cell number, supporting the view that androgens are involved in regulating Sertoli cell proliferation/number in fetal life.

It was initially hoped that exposing animals to flutamide would be the most straightforward way of determining what role, if any, androgens play in the intratesticular changes seen in DBP-exposed animals, and in particular their involvement in Sertoli cell proliferation/number. Although there are no obvious reasons why flutamide, or any other treatment, would not reach the testicular cells (the blood-testis barrier has not formed at this age), it became apparent that although flutamide had very pronounced effects at peripheral sites of androgen action, namely reduction of the male AGD to that of a typical female, it had no obvious effect on the testis when administered on its own. There are two possible, and opposing, explanations for this. The first, is that flutamide does not cause any changes to the endpoints evaluated because none of them are androgen-dependent. The second, is that levels of testosterone within the testis are too high for flutamide to effectively antagonize them. Whilst the former possibility cannot be ruled out, there are two pieces of data that support the latter interpretation. First, the dose of flutamide used for the present studies failed to induce regression of the Wolffian duct in the exposed males (Welsh et al., 2006), consistent with the higher local testosterone levels in this duct compared with at peripheral sites

(e.g. prostate, perineum), and testosterone levels within the testis are presumably even higher than in the Wolffian duct. Second, combined exposure to DBP + flutamide induced a 17.1% larger decrease in Sertoli cell number than did exposure to DBP alone, which may mean that flutamide is marginally effective when intratesticular testosterone levels are markedly subnormal due to DBP treatment. An additional possibility is that flutamide exposure elevates endogenous FSH levels in male fetuses, as it does neonatally in rats (Atanassova et al., 2005), which would presumably increase Sertoli cell proliferation and thus counteract any anti-androgenic effect of flutamide on this parameter.

It has already been well established that exposure to DBP results in a reduction in intratesticular testosterone (Fisher et al., 2003; Mylchreest et al., 1998; Mylchreest et al., 1999; Mylchreest et al., 2000) and, in postnatal life, a reduced AGD (Carruthers and Foster, 2005), and our present data confirmed this, although the reduction in AGD evident at e21.5 in the present studies was slight and not always statistically significant. All of the groups exposed to either DBP alone, or with a co-treatment, showed a reduction in intratesticular testosterone levels and a concurrent and equivalent reduction in Sertoli cell number. It cannot be excluded that DBP gave rise to this reduction in Sertoli cell number via a non-testosterone dependent mechanism. However, if this were the case it would fail to account for the reduction in Sertoli cell number observed in ARKO (chapter 3) and *tfm* mice (Johnston et al., 2004) and the strong correlation between Sertoli cell number and intratesticular testosterone levels across a wide testosterone range as observed presently. Consequently, the present findings on Sertoli cell number are most plausibly explained by the change in intratesticular testosterone levels.

Our primary intention in treating with TP was to ascertain which, if any, of the DBP effects could be prevented if the DBP-induced reduction in intratesticular testosterone levels were attenuated, thus confirming that they were testosterone-dependent. Surprisingly, TP did not reverse any of the DBP-induced effects, although it was able to successfully masculinise AGD, induce prostate formation and stabilise the Wolffian duct in exposed females, and to some extent increase AGD in males, when administered alone or with DBP. Therefore, whilst this dose of TP was clearly biologically active at more peripheral sites, it was unable to restore intratesticular testosterone levels in DBP-exposed animals to normal. In fact, animals exposed to DBP + TP had lower intratesticular testosterone than those exposed to DBP alone and treatment with TP alone also resulted in a (non-significant) reduction in intratesticular testosterone levels. The most logical interpretation of these findings is that TP treatment caused a reduction in LH levels via increased negative feedback at the hypothalamic-pituitary axis (Carruthers and Foster, 2005; Ramaswamy et al., 2000), and thus reduced LH drive to testosterone production, and consequently this exacerbated the reduction in intratesticular testosterone levels caused by DBP alone. Exposure to DBP + TP resulted in the greatest reduction in Sertoli cell number, but also caused quite marked decreases in fetal bodyweight, consistent with previous reports (Wolf et al., 2004), so it is also possible that growth restriction may have contributed to the effect on Sertoli cell number.

DMBA treatment was undertaken in response to published *in vitro* evidence which suggested that it could antagonise testosterone action distal to AR-binding (Kizu et al., 2003; Vinggaard et al., 2000), thus providing a credible explanation for the presumed reduction in Sertoli cell number in men whose mothers smoked during pregnancy and who display a pronounced decrease

in testis size and sperm counts in adulthood (Jensen et al., 2004; Storgaard et al., 2003). However, DMBA exposure did not induce any significant effects on measured parameters, including Sertoli cell number, when administered on its own, and co-treatment with DBP + DMBA did not obviously exacerbate any of these parameters compared with DBP treatment alone. Therefore, at the dose used in this study, no evidence was obtained to support the hypothesis that DMBA antagonises androgen action either peripherally or intratesticularly. In fact, other published data has demonstrated that *in utero* exposure to 2,3,7,8-Tetracholrodibenzo-p-dioxin (TCDD), which like DMBA acts through the aryl hydrocarbon receptor, does not impair the androgen receptor pathway (Ko et al., 2004), contrary to earlier *in vitro* evidence.

4.4.1.2 DBP dose response

The purpose of this study was to determine whether fetal rats would display a dose dependent reduction in testiscular testosterone and Sertoli cell number, when exposed to 500, 100, 20 or 4mg/kg DBP, from e13.5-20.5. The results from this study demonstrated that although doses of 20 and 4mg/kg DBP had no significant effect on intratesticular testosterone, DBP 500mg/kg caused a greater reduction in testiscular testosterone than did DBP 100mg/kg, as was expected. As shown previously in this chapter, DBP 500mg/kg also caused a reduction in Sertoli cell number. However, contrary to expectation, treatment with DBP 100mg/kg did not cause any significant change in Sertoli cell number at e21.5, despite significantly reducing testicular testosterone levels.

Although this result was unexpected and contradicted earlier evidence demonstrating reductions in testicular testosterone coincident with

reductions in Sertoli cell number, it did raise the possibility that levels of intratesticular testosterone, in control fetal males, are in excess of those actually required to regulate Sertoli cell proliferation. Consequently, levels of testicular testosterone can be reduced, to at least those resulting from DBP 100mg/kg exposure, and still be sufficient to drive Sertoli cell proliferation. This would also suggest that there may be a maximum threshold level of testicular testosterone, beyond which Sertoli cell proliferation can increase no further.

4.4.1.3 DBP treatment windows

This study set out to examine whether androgens act to modulate Sertoli cell proliferation during specific time windows in development, or throughout the whole of the later stages of gestation. To investigate this, pregnant dams were treated with DBP 500mg/kg, during the following gestational time windows; from e13.5-15.5 (early), e15.5-17.5 (middle), e19.5-20.5 (late), e13.5-20.5 (standard) or e11.5-20.5 (extended), and analysed for testicular testosterone levels, AGD and Sertoli cell numbers at e21.5. Intratesticular testosterone and Sertoli cell number were also investigated at e17.5, in control and DBP-exposed animals, to establish whether DBP reduced testicular testosterone and Sertoli cell number earlier in development. Sertoli cell proliferation indices were also determined at e17.5 and e21.5 in control and DBP treated animals, to ascertain normal rates of Sertoli cell proliferation and whether this was altered after DBP exposure.

The data demonstrated that intratesticular testosterone was significantly reduced after exposure to DBP in the late, standard and extended treatment windows, and that Sertoli cell number was also significantly reduced after treatment during these time windows. This data is consistent with the

hypothesis that testosterone plays a role in Sertoli cell proliferation, as discussed earlier in section 4.4.1.1. Exposure to DBP during the early and middle treatment windows, however, had no effect on either testicular testosterone or Sertoli cell number, measured at e21.5. Since testosterone production does not commence until e15.5 (Warren et al., 1973), a reduction in testicular testosterone, and a consequent reduction in Sertoli cell number, was not anticipated in the early treatment window (e13.5-e15.5). However, this argument would not apply to the middle treatment window (e15.5-e17.5), especially as other studies in our Group have established that this time window is the most important for 'masculinisation' of the peripheral reproductive system in male rats (Welsh et al., 2007). Analysis of testicular testosterone and Sertoli cell number was not performed until e21.5, which raised the possibility that any reduction in testicular testosterone and Sertoli cell number that occurred as a result of the middle window of DBP exposure, was not evident at e21.5 because the testis had undergone a period of recovery. This interpretation was further supported by data collected from pups exposed to DBP from e13.5 to e16.5 and then killed on e17.5. These animals demonstrated a significant 53% reduction in intratesticular testosterone and a 52% reduction in Sertoli cell number at e17.5, though due to high between-animal variation, this reduction was not statistically significant. This is consistent with published data that demonstrated that the reduction in fetal testosterone levels caused by DBP exposure was reversible and that testosterone levels begin to recover 24 hours after exposure has ceased (Thompson et al., 2004), offering a possible mechanism for the recovery in Sertoli cell number seen after exposure to the 'middle' DBP treatment window. A recovery in Sertoli cell number has also been identified after birth, with males exposed to DBP for the full (standard) treatment

window exhibiting normal Sertoli cell numbers by postnatal day 25 – 90 (Hutchison et al., 2007). The mechanism for this recovery is unknown, since data from our Group and (Lee et al., 1975), has demonstrated that both FSH and testosterone remain low for most of this period, indicating that there must be another factor/mechanism capable of stimulating Sertoli cell proliferation.

Sertoli cell proliferation indices were also calculated at e17.5 and e21.5 in animals that had been exposed to DBP from e13.5 until the day prior to kill. Rates of Sertoli cell proliferation in control animals at e17.5 (22.9%) and e21.5 (27.4%) were in a similar range to those previously reported as being 16% at gestational day 16 and 26.8% at gestational day 20 (Orth, 1982). This publication also described Sertoli cell proliferation from the earliest age investigated (gestational day 16), reported the peak in the Sertoli cell proliferation index at gestational day 20, and noted that the expansion of the Sertoli cell population coincided with the increasing production of testosterone (Orth, 1982). DBP-exposure induced a significant reduction in the Sertoli cell proliferation index at e21.5, which reflects the reduction in Sertoli cell number seen at that age. No significant reduction in Sertoli cell proliferation was seen at e17.5 however, which was surprising since although the reduction in Sertoli cell number at this age was not significant, most likely due to between-animal variation, it was 52%. A possible explanation for the greater reduction in Sertoli cell proliferation seen at e21.5, is that DBP exposure also affects FSH, a factor that is known to stimulate Sertoli cell proliferation and is first detected at e19.5 (Majdic et al., 1997). In fact, it has been demonstrated that exposing cultured rat Sertoli cells to mono-(2-ethylhexyl)phthalate (MEHP) inhibited FSH action at the level of the FSH receptor (Lloyd and Foster, 1988), and neonatal Sertoli cells cultured with

MEHP exhibited reduced basal and FSH-stimulated Sertoli cell proliferation (Li et al., 1998).

AGD was significantly reduced in male pups exposed to DBP during the middle, standard and extended treatment windows, and these results are consistent with previous data that has demonstrated that e15.5-e17.5 is the critical window for androgens to mediate AGD masculinisation, as well as prostate formation and Wolffian duct development (Welsh et al., 2007). Although AGD was not measured in males treated during the late window, earlier studies, involving the administration of flutamide during this window, demonstrated no effect on AGD (Welsh et al., 2007).

4.4.2 *In vitro* studies

The aim of these studies was to determine whether the Sertoli cell proliferation rates of e19.5 testis explants, cultured for 48 hours in the presence of a variety of chemicals, could be modulated. The principal chemical investigated was hydroxyflutamide, a competitive androgen receptor antagonist, expected to block/limit androgen action and thus reduce Sertoli cell proliferation rates/indices. Testis explants were also cultured with factors expected to increase Sertoli cell proliferation; FSH, hCG and EGF, and with cyclopamine, an inhibitor of hedgehog signalling, expected to disrupt communication between the Sertoli cells and peritubular myoid cells, and to delay Leydig cell differentiation.

The present data demonstrated that culturing testis explants taken from e19.5 untreated pups, with either 10^{-4}M, 10^{-5}M or 10^{-6}M hydoxyflutamide, had no significant effect on Sertoli cell proliferation indices. This result is surprising since the tissue architecture of the testis explants remained largely intact, and Sertoli cell proliferation indices averaged 30%, implying that the testis

explants were viable. There are several possible explanations for why a reduction in Sertoli cell proliferation rates was not observed with hydroxyflutamide. *In vivo* exposure to flutamide failed to induce a reduction in Sertoli cell number (discussed earlier in 4.4.1.1), which could suggest that androgen action is in fact not required for Sertoli cell proliferation and the reduction seen with DBP-exposure is due to some other toxic effect on the Sertoli cell. As discussed previously, however, this would not explain the reduction in Sertoli cell number seen in ARKO mice (chapter 3). Another possibility is that the dose of hydroxyflutamide administered to the cultures were too low to effectively antagonise the high levels of testosterone produced within the cultured testes. Overall, this negative finding is puzzling and requires further investigation. Ideally, more *in vitro* studies would be performed, using testes from a variety of fetal ages and culturing with higher doses of hydroxyflutamide or with an inhibitor of testosterone production, for example, ketoconazole. However, previous attempts, in our Group, to reduce testosterone production in e19.5 testis explants using ketoconazole, showed massive variation and inconsistent results (Hallmark et al., 2007).

Having failed to reduce Sertoli cell proliferation rates *in vitro*, cultures were then set up with factors expected to increase Sertoli cell proliferation. Testis explants were cultured for 48 hours with FSH, hCG, FSH + hCG or EGF and Sertoli cell proliferation indices calculated. Unexpectedly, the data from these cultures demonstrated that none of these treatments induced a significant increase in Sertoli cell proliferation rate. FSH and EGF had been selected due to previous evidence (Allan et al., 2004; Johnston et al., 2004; Meachem et al., 1996; Petersen et al., 2001) that strongly suggested they would act to increase Sertoli cell proliferation, however, this evidence was either established using

in vivo exposure, or with *in vitro* cultures of postnatal testes, which may not be comparable to these present studies. hCG was selected as earlier studies in our Group had demonstrated that it increased testosterone production, by fetal rat testes, *in vitro* (Hallmark et al., 2007). It must also be taken into account however, that Sertoli cell proliferation has been reported to be at its maximum level, 26.8%, at gestational day 20 (Orth, 1982), and that Sertoli cell proliferation indices in these studies have been approximately 30%, so another possible explanation for not seeing an increase in Sertoli cell proliferation, could be that the system was already running at its maximum, so cannot be increased further, regardless of the factors/chemicals it was exposed to.

Testis explants were also cultured with cyclopamine, a chemical known to disrupt Dhh signalling. Testis explants cultured with cyclopamine demonstrated a significant reduction in both testosterone production, determined by measuring testosterone levels in the culture media, and a significant reduction in the Sertoli cell proliferation index. This reduction in testosterone production is not surprising, as it has previously been demonstrated that cyclopamine exposure results in incomplete Leydig cell differentiation (Yao et al., 2002) and the majority of mice lacking Dhh are under-masculinised (Clark et al., 2000). The concurrent reductions in both testosterone production and Sertoli cell proliferation rates, further supports the hypothesis that testosterone plays a role in Sertoli cell proliferation.

The ideal treatment to compare the *in vivo* and *in vitro* system would have been MBP, the main metabolite of DBP. However, Nina Hallmark had previously shown that MBP had no effect on Sertoli cell proliferation or testosterone production *in vitro* using e19.5 testis explants (Hallmark et al.,

2007). Other *in vitro* studies have been more successful however, and it has been demonstrated that fetal and neonatal organ cultures of rat testes exposed to MEHP, the metabolite of Di(2-ethylhexyl)phthalate (DEHP), exhibit reduced Sertoli cell proliferation at both e18 and postnatal day 3 (Li and Kim, 2003)

The main shortfall of the *in vitro* system was that actual Sertoli cell number per explant could not be calculated due to 1) variable size of each explant and 2) variable occurrence of small necrotic areas of tissue. Consequently, instead of seeing the cumulative effects of exposure to a treatment over a period of time on cell number, the cell proliferation index only represents a 'snap shot' of the effect during the 2 hour period that the BrdU was present in the culture medium. The reduction in Sertoli cell proliferation over two hours may be minimal, but if this continued for 48 hours or longer, the overall effect on Sertoli cell number could be significant, making Sertoli cell number a far more sensitive measure than proliferation indices.

The number of cultures performed for these studies were not extensive, so although conclusions need to be tentative, it could be inferred that Sertoli cell proliferation is so important that multiple mechanisms exist to ensure that it proceeds normally, suggesting that there may be other unknown factors able to compensate when the system is disrupted. If this is the case, then it raises questions as to how DBP is so successful at reducing Sertoli cell number *in vivo*, although the most plausible explanation for that would be that DBP affects the testis through multiple pathways, possibly affecting the Leydig cells, Sertoli cells and germ cells.

Establishing whether androgens play a role in Sertoli cell proliferation is a vital issue that needs to be resolved, since it has considerable physiological

and human health (TDS) implications. Overall the results from this chapter are consistent with the hypothesis that androgens regulate Sertoli cell proliferation, coherent with present and published observations in ARKO mice (chapter 3 and (Tan et al., 2005). However, there were some inconsistencies, and the principal weakness of studies in this chapter was that the only treatment to successfully reduce Sertoli cell number was DBP, and although exposure to DBP also causes a major reduction in testicular testosterone, it may also have directly affected the Sertoli cells, as has previously been shown to be the case postnatally (Lloyd and Foster, 1988). In order to rule out the possibility that DBP instigates the reduction in Sertoli cell number via a direct toxic effect on the Sertoli cells and not as a result of suppressed testicular testosterone levels, another treatment that reduces testicular testosterone, but has no known toxic effects on the Sertoli cells, needs to be examined, and this will be addressed with the administration of dexamethasone in chapter 6.

5 Androgens and testicular dysgenesis

5.1 Introduction

Following on from chapters 3 and 4, in which evidence was presented to suggest that androgens play a role in Sertoli cell proliferation, evidence has also emerged from the DBP rat model for testicular dysgenesis syndrome (TDS) that suggests reduced androgen action may play a role in testicular dysgenesis. The likelihood of this is reinforced by patients with complete androgen insensitivity syndrome (CAIS; the human equivalent of *tfm* mice), in whom focal areas of testicular dysgenesis have been reported (Hannema et al., 2006). These patients are also at a higher risk of developing testicular germ cell cancer (Cools et al., 2005; Verp and Simpson, 1987).

In utero exposure of rats to DBP induces a marked reduction in testicular testosterone production, widespread occurrence of multinucleated fetal germ cells (Fisher et al., 2003; Parks et al., 2000; Shultz et al., 2001) and focal dysgenesis (Fisher et al., 2003; Mahood et al., 2005; Mahood et al., 2006). Areas of focal dysgenesis, which comprise malformed seminiferous tubules with intratubular Leydig cells and immature Sertoli cells are not present in the fetal testes and only become apparent in postnatal life (Fisher et al., 2003; Mahood et al., 2005), however, abnormally large clusters/aggregations of Leydig cells can be seen centrally in the fetal DBP exposed testes and are thought to give rise to the dysgenetic areas evident in postnatal life (Mahood et al., 2006). The studies in this chapter sought to determine whether reduced androgen levels contribute causally to multinucleated gonocyte formation or Leydig cell aggregation (used as a surrogate for focal dysgenesis) in fetal life in DBP-exposed animals or in animals exposed to other treatments expected

to manipulate androgen levels (TP) or action (flutamide, DMBA), and whether these dysgenetic endpoints also occur in ARKO or *tfm* mice.

5.2 Materials and Methods

In brief, time-mated pregnant dams were treated daily using one of the regimes shown in Table 5.1. All pups were killed at e21.5.

Table 5.1 Summary of *in utero* treatment regimes used.

Treatment	Treatment window
Control (vehicle only)	e13.5-20.5
Flutamide 100μg/kg	e13.5-20.5
DMBA 400μg/kg	e13.5-20.5
TP 20mg/kg	e13.5-20.5
DBP 500mg/kg	e13.5-20.5
DBP 500mg/kg + Flutamide 100mg/kg	e13.5-20.5
DBP 500mg/kg + DMBA 400μg/kg	e13.5-20.5
DBP 500mg/kg + TP 20mg/kg	e13.5-20.5
DBP 500mg/kg	e13.5-15.5
DBP 500mg/kg	e15.5-17.5
DBP 500mg/kg	e19.5-20.5
DBP 500mg/kg	e13.5-20.5
DBP 500mg/kg	e11.5-20.5

The pregnant dams were killed, the pups were recovered, and the testes were removed and then trimmed (section 2.4). After fixation (section 2.4.6), processing (section 2.4.7) and sectioning (section 2.6.1.1), testes were stained with either toluidine blue (section 2.6.1.9), AMH or for 3β-HSD expression (section 2.6.1), and the percentage of seminiferous cords containing multinucleated gonocytes determined (sections 2.7.5) or Leydig cell aggregation analysed (section 2.7.4). Testes from control rats or rats exposed to DBP from e13.5-21.5 and e11.5-21.5 and then left to reach adulthood were also sectioned and stained with SMA to allow the identification of postnatal

focal dysgenetic areas. The number of animals used is indicated in each figure legend. Animals came from a minimum of three litters in all cases.

5.2.1 Statistical analysis

Data were analysed using one-way ANOVA followed by the Bonferroni post test, except when comparing the frequency of multinucleated gonocytes and occurrence of small, medium and large Leydig cell clusters in the *tfm* and wild type mice. These data were analysed using Student's unpaired *t* test.

5.3 Results

5.3.1 Occurrence of multinucleated gonocytes

Sections of testis from e21.5 rats treated *in utero* with either corn oil (control), flutamide, DMBA, TP, DBP, DBP + flutamide, DBP + DMBA or DBP + TP, were counterstained with toluidine blue to enable the clear visualisation and enumeration of any multinucleated gonocytes present within the seminiferous cords. All treatment groups, including the control group, exhibited some multinucleated gonocytes, although they were detected with much greater frequency in those animals exposed *in utero* to DBP, either alone or combined with another treatment (Figure 5.1).

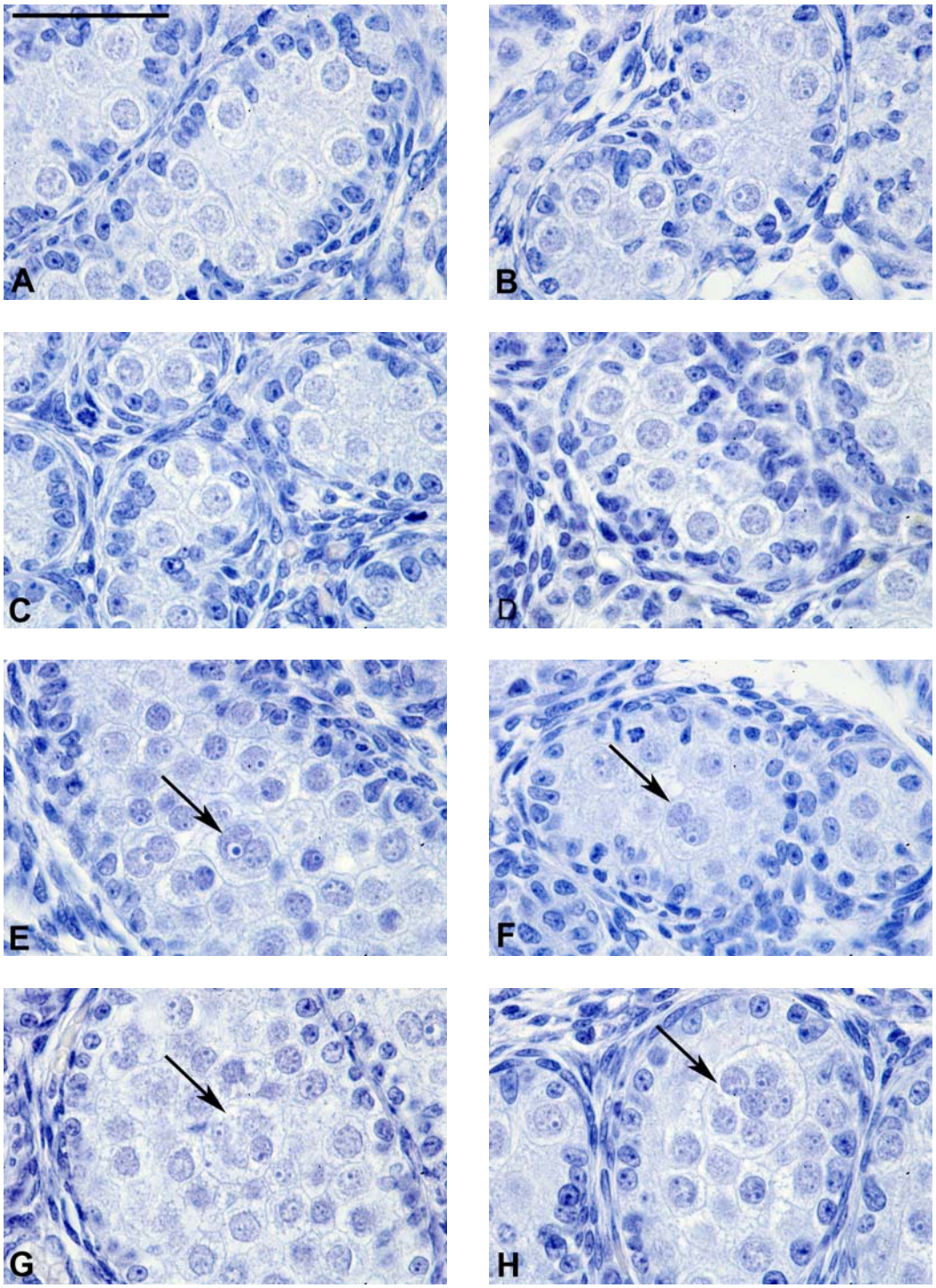

Figure 5.1 Photomicrograph illustrating the occurrence of multinucleated gonocytes (arrows) in e21.5 rats exposed to (A) corn oil, (B) flutamide, (C) DMBA, (D) TP, (E) DBP, (F) DBP + flutamide, (G) DBP + DMBA or (H) DBP + TP. Sections immunostained for AMH. Scale bar shows 50μm.

In utero exposure to DBP resulted in a 44-fold increase in the percentage of cords exhibiting multinucleated gonocytes, and combined treatment with

DBP plus either flutamide, DMBA or TP caused a similar level of increase in multinucleated gonocytes (Figure 5.2). Exposure to flutamide, DMBA or TP alone did not produce a significant increase in the occurrence of multinucleated gonocytes compared with controls (Figure 5.2).

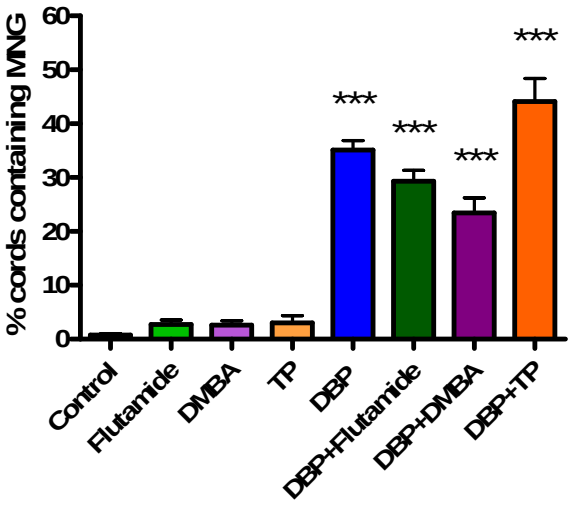

Figure 5.2 The percentage of seminiferous cords exhibiting multinucleated gonocytes at e21.5 after *in utero* exposure to flutamide (n=9), DMBA (n=7), TP (n=8), DBP (n=10), DBP + flutamide (n=15), DBP + DMBA (n=9), DBP + TP (n=8) or vehicle alone (control; n=24). Values are means ± SEM. ***p<0.001 in comparison to control value. None of the treatments combined with DBP induced effects significantly different from treatment with DBP alone.

5.3.2 Leydig cell clusters

Changes in Leydig cell distribution were clearly visible in all DBP-exposed treatment groups when compared to controls (Figure 5.3), with the result that abnormally large Leydig cell clusters were present in the centre of the testes and there was a corresponding decrease in the number of small Leydig cell clusters. Leydig cell distribution in testes from animals exposed to flutamide, DMBA or TP alone were not obviously different from control sections (Figure 5.3).

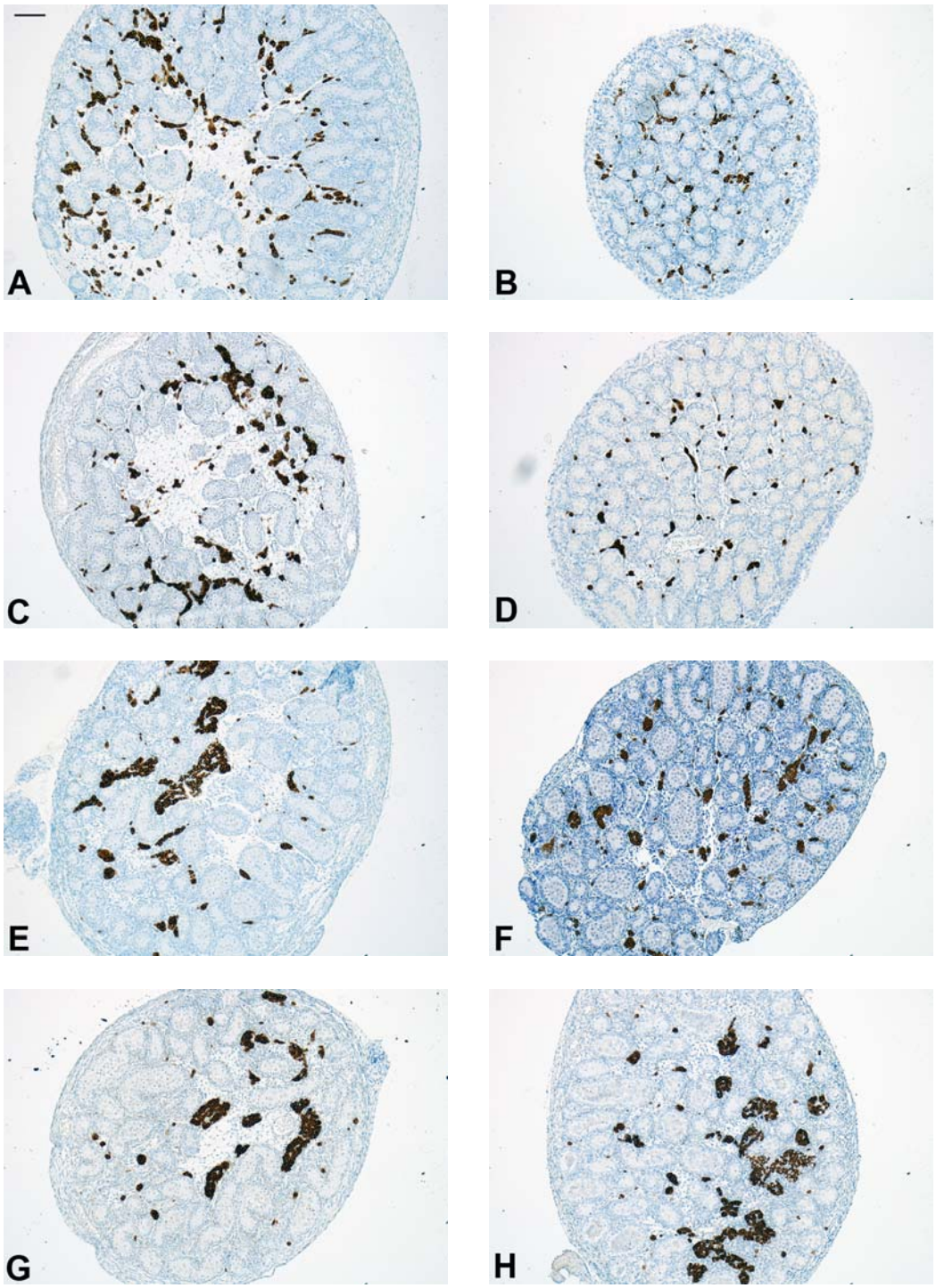

Figure 5.3 Representative photomicrographs illustrating the distribution of Leydig cells (3β-HSD-positive, brown) in e21.5 rats exposed to corn oil (control; A), flutamide (B), DMBA (C), TP (D), DBP (E), DBP + flutamide (F), DBP + DMBA (G) or DBP + TP (H). Changes in Leydig cell distribution were seen in all animals exposed to DBP alone, or in combination with other treatments. Scale bar shows 50μm.

Quantification of Leydig cell aggregation by cluster analysis revealed a significant reduction in the percentage of total Leydig cell cluster area accounted for by small Leydig cell clusters in animals exposed to DBP alone or DBP combined with flutamide, DMBA or TP (Figure 5.4).

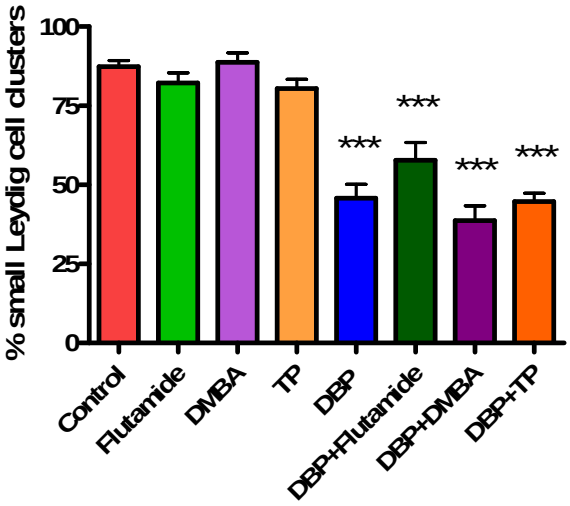

Figure 5.4 The occurrence of small Leydig cell clusters in the testes of e21.5 rats exposed *in utero* to corn oil (control; n=8), flutamide (n=5), DMBA (n=4), TP (n=8), DBP (n=8), DBP + flutamide (n=5), DBP + DMBA (n=5) or DBP + TP (n=8). Values are means ± SEM. ***p<0.001 in comparison to control value. None of the treatments combined with DBP induced effects significantly different from treatment with DBP alone.

Leydig cell clusters of medium size were evident in all treatment groups (Figure 5.5).

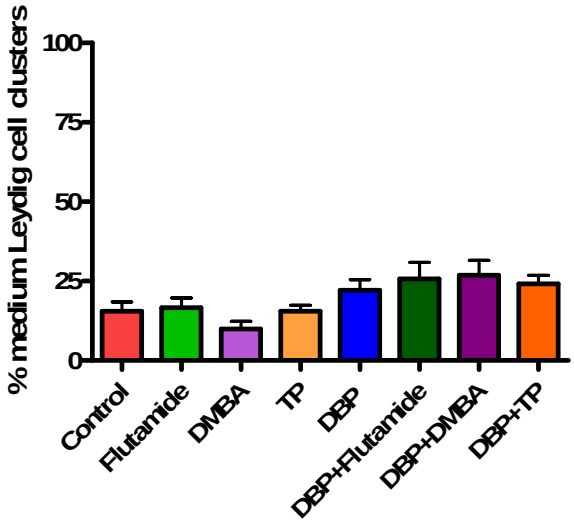

Figure 5.5 The occurrence of medium Leydig cell clusters in the testes of e21.5 rats exposed *in utero* to corn oil (control; n=8), flutamide (n=5), DMBA (n=4), TP (n=8), DBP (n=8), DBP + flutamide (n=5), DBP + DMBA (n=5) or DBP + TP (n=8).

Large Leydig cell clusters were not detected in controls but were detected in all treatment groups. However, the induction of large Leydig cell clusters was only significant in animals exposed to DBP (32%), DBP + DMBA (34%) or DBP + TP (31%; Figure 5.6).

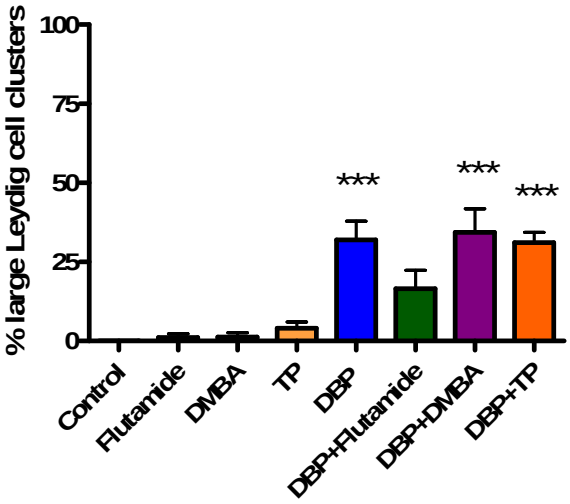

Figure 5.6 The occurrence of large Leydig cell clusters in the testes of e21.5 rats exposed *in utero* to corn oil (control; n=8), flutamide (n=5), DMBA (n=4), TP (n=8), DBP (n=8), DBP + flutamide (n=5), DBP + DMBA (n=5) or DBP + TP (n=8). Values are means ± SEM. ***$p<0.001$ in comparison to control value. None of the treatments combined with DBP induced effects significantly different from treatment with DBP alone.

5.3.3 Androgen dependence of dysgenetic endpoints

Potential relationships/correlations between the intratesticular levels of testosterone and testicular dysgenetic endpoints were evaluated by plotting the mean intratesticular testosterone values (expressed as a percentage of intra-assay control) versus occurrence of multinucleated gonocytes and percentage of large Leydig cell clusters. As the latter were not measured in the same animals as those in which the testosterone levels were evaluated, only group mean values were used for analysis. There was a negative correlation between intratesticular testosterone levels and the occurrence of multinucleated gonocytes (Figure 5.7A), as well as between intratesticular testosterone levels and the percentage of large Leydig cell clusters (Figure 5.7B).

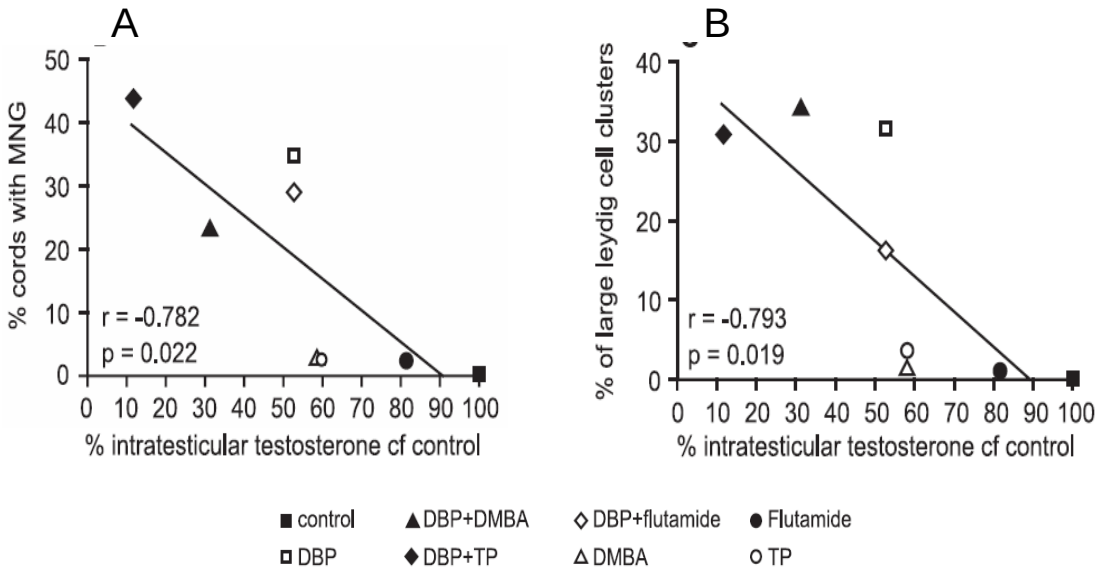

Figure 5.7 Correlations at e21.5 between intratesticular testosterone levels and percentage of cords exhibiting multinucleated gonocytes (A) and percentage of large Leydig cell clusters (B). Each data point represents the mean value for each treatment group. Intratesticular testosterone levels have been expressed as the percentage of the mean control value in the same testosterone assay (chapter 4).

It is impossible to determine, from this data, whether the 'relationships' between intratesticular testosterone levels and multinucleated gonocytes or Leydig cell aggregation are real/causal or coincidental, since DBP exposure may induce either or both of these endpoints independently of the reduction in intratesticular testosterone. Consequently, other methods of investigating the role that reduced androgens may play in testicular dysgenesis were sought. In chapter 4 it was demonstrated that DBP exposure during different treatment windows had different effects on testicular testosterone levels, therefore testes taken from rats exposed to DBP during different treatment windows could be used to investigate multinucleated gonocytes and Leydig cell aggregation, and to determine whether or not they occur during treatment windows when testicular testosterone is reduced. The *tfm* mice also provide an ideal model to investigate whether multinucleated gonocytes and/or Leydig cell aggregates result from reduced/ablated androgen action, and at e18.5, are developmentally comparable to the e21.5 rat testes investigated earlier in the chapter.

5.3.4 DBP treatment windows

Although multinucleated gonocytes occurred at low frequency in the control, e13.5-15.5 and e15.5-17.5 treatment groups, they occurred much more frequently and were therefore more obvious at a glance, in testes exposed to DBP during the e19.5-20.5, e13.5-20.5 and e11.5-20.5 treatment windows (Figure 5.8).

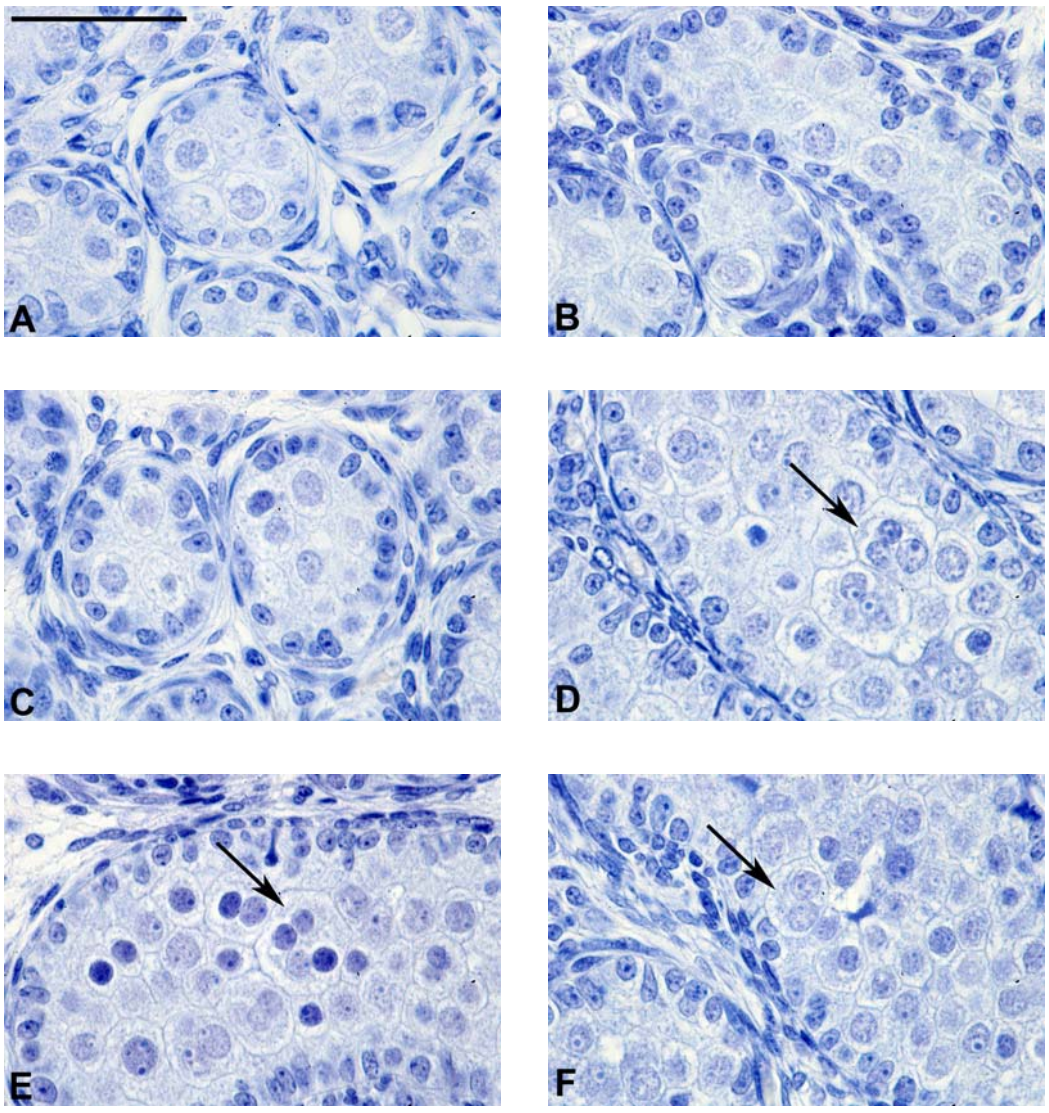

Figure 5.8 Representative photomicrographs demonstrating the occurrence of multinucleated gonocytes (arrows) in e21.5 rat testes treated with DBP from e13.5-15.5 (B), e15.5-17.5 (C), e19.5-20.5 (D), e13.5-20.5 (E), e11.5-20.5 (F) or in controls treated with vehicle alone from e13.5-20.5 (A). Sections are stained with haemotoxylin. Scale bar represents 50μm.

The percentage of cords containing multinucleated gonocytes was significantly increased in testes exposed to DBP from e19.5-20.5, e13.5-20.5 and e11.5-20.5 (Figure 5.9). The differences in occurrence of multinucleated gonocytes seen between the animals treated from e19.5-20.5 and those treated from e13.5-e21.5, was significant ($p<0.001$), as was the difference seen between the animals treated from e13.5-e20.5 and those treated from e11.5-e20.5 ($p<0.001$).

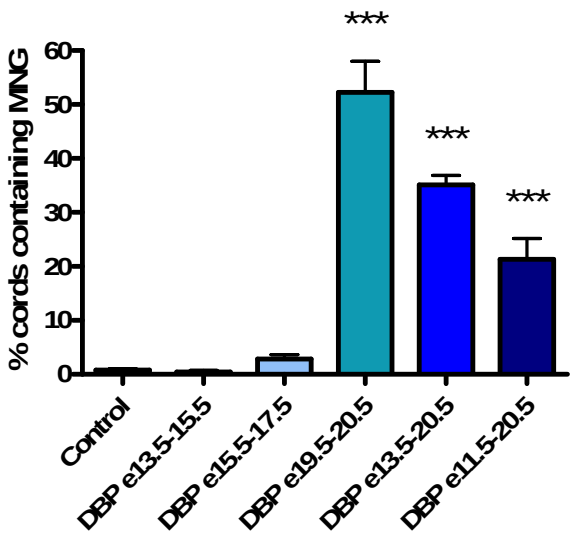

Figure 5.9 Effect of *in utero* exposure to DBP 500mg/kg during the following treatment windows; e13.5-15.5 (n=7), e15.5-17.5 (n=6), e19.5-20.5 (n=6), e13.5-20.5 (n=10), e11.5-20.5 (n=5) or with vehicle alone during the standard e13.5-20.5 treatment window (control; n=24) on the percentage of seminiferous cords exhibiting multinucleated gonocytes (MNG) at e21.5. Values are means ± SEM. ***p<0.001 in comparison to control value.

Changes in Leydig cell distribution were clearly visible in testes exposed to DBP from e13.5-20.5 and e11.5-20.5 (Figure 5.10). In these testes there were fewer small Leydig cell clusters and an increase in large Leydig cell clusters. The distribution of Leydig cell clusters were not obviously different in the testes from animals exposed to DBP from e13.5-15.5, e15.5-17.5 or e19.5-20.5 (Figure 5.10).

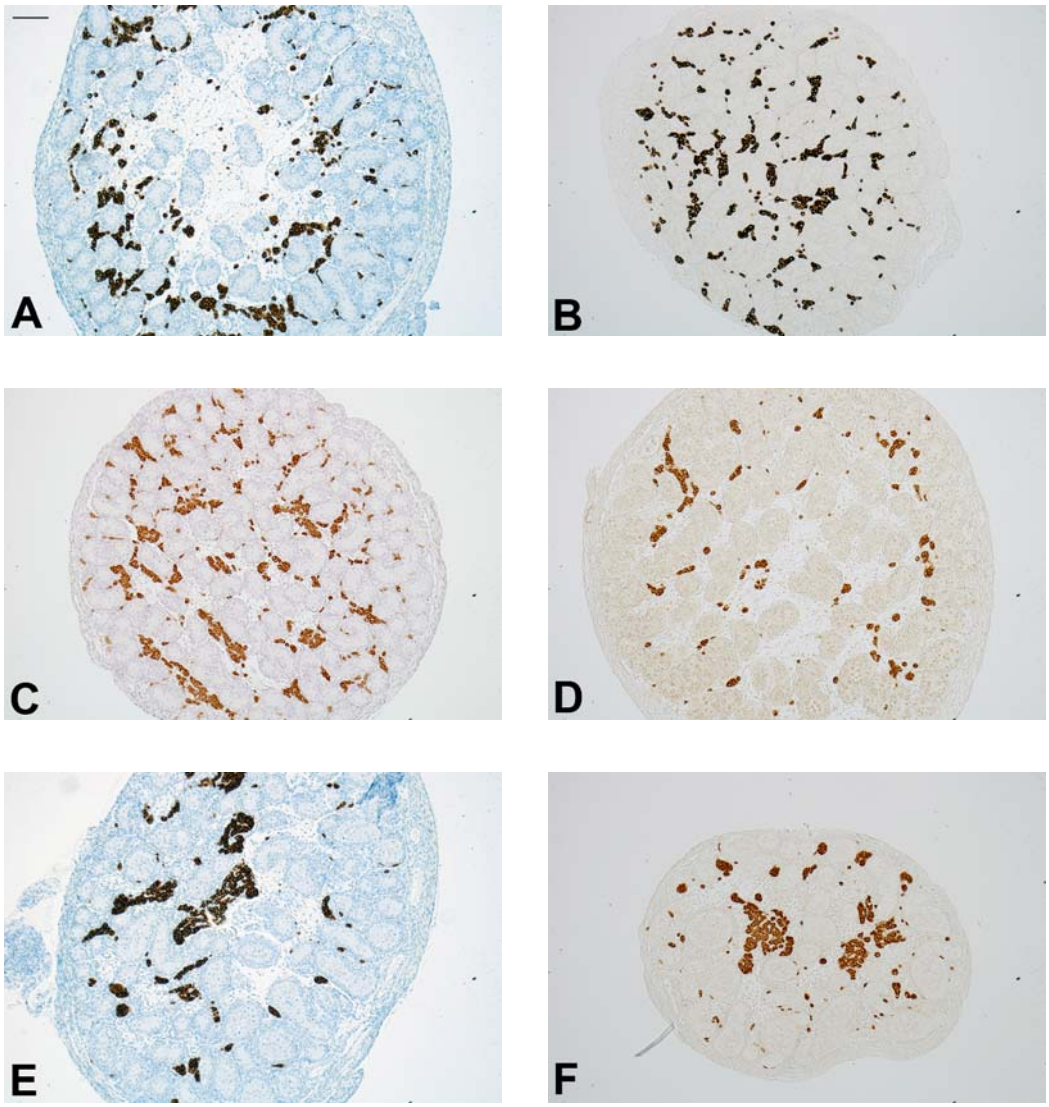

Figure 5.10 Representative photomicrographs demonstrating the distribution of Leydig cells (immunostained for 3β-HSD) in the testes of e21.5 rats exposed to (A) vehicle alone from e13.5-20.5 (control), or (B) DBP 500mg/kg from e13.5-15.5, (C) e15.5-17.5, (D) e19.5-20.5, (E) e13.5-20.5 or (F) e11.5-20.5. Leydig cell distribution was altered in animals exposed to DBP from e13.5-20.5 or e11.5-20.5. Scale bar represents 100μm.

Quantitative cluster analysis of Leydig cell aggregation demonstrated a significant reduction in the percentage of total Leydig cell cluster area accounted for by small Leydig cell clusters in animals exposed to DBP from e19.5-20.5, e13.5-20.5 or e11.5-20.5 (Figure 5.11).

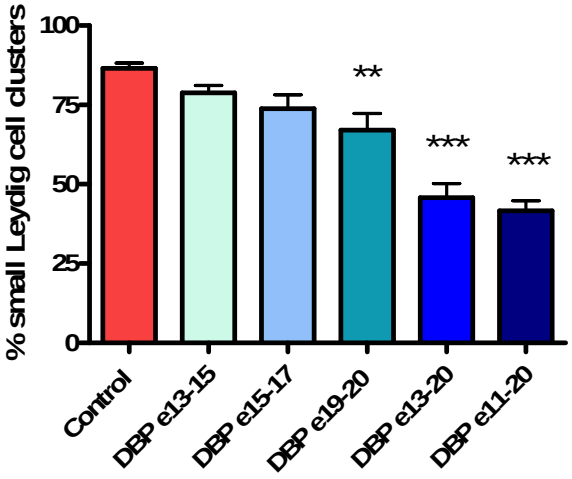

Figure 5.11 The occurrence of small Leydig cell clusters in the testes of e21.5 rats exposed *in utero* to DBP 500mg/kg during the following treatment windows; e13.5-15.5 (n=6), e15.5-17.5 (n=6), e19.5-20.5 (n=6), e13.5-20.5 (n=8), e11.5-20.5 (n=5) or with vehicle alone during the standard e13.5-20.5 treatment window (control; n=10). Values are means ± SEM. **p<0.01, ***p<0.001 in comparison to control value.

Leydig cell clusters of medium size were evident in all treatment groups (Figure 5.12).

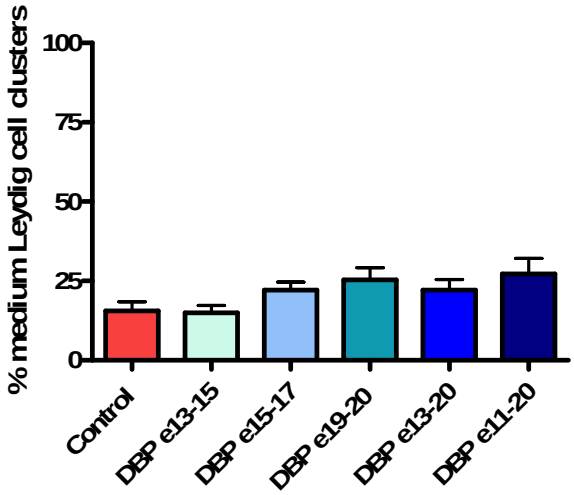

Figure 5.12 The occurrence medium Leydig cell clusters in the testes of e21.5 rats exposed *in utero* to DBP 500mg/kg during the following treatment windows; e13.5-15.5 (n=6), e15.5-17.5 (n=6), e19.5-20.5 (n=6), e13.5-20.5 (n=8), e11.5-20.5 (n=5) or with vehicle alone during the standard e13.5-20.5 treatment window (control; n=10).

Large Leydig cell clusters were not detected in controls but were detected in all animals exposed to DBP during the various treatment windows. However, the induction of large Leydig cell clusters was only statistically significant in animals exposed to DBP from e13.5-e210.5 (32%) or from e11.5-20.5 (31%; Figure 5.13).

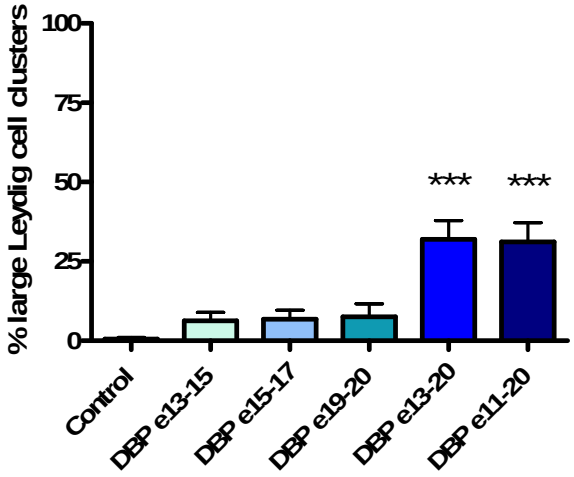

Figure 5.13 The occurrence large Leydig cell clusters in the testes of e21.5 rats exposed *in utero* to DBP 500mg/kg during the following treatment windows; e13.5-15.5 (n=6), e15.5-17.5 (n=6), e19.5-20.5 (n=6), e13.5-20.5 (n=8), e11.5-20.5 (n=5) or with vehicle alone during the standard e13.5-20.5 treatment window (control; n=10). Values are means ± SEM. ***p<0.001 in comparison to control value.

5.3.5 Fetal Leydig cell aggregation precedes postnatal focal dysgenesis

As fetal Leydig cell aggregation has been used as a presumed precursor to postnatal focal dysgenesis, the relationship between the two endpoints was investigated further. As has been shown previously in this chapter, large Leydig cell clusters occur with high frequency (32%) in fetal testes exposed to DBP from e13.5-20.5, but are found at low frequency (8%) in testes exposed to DBP from e19.5-20.5. There is great variability between animals in both DBP treatment groups (Figure 5.14).

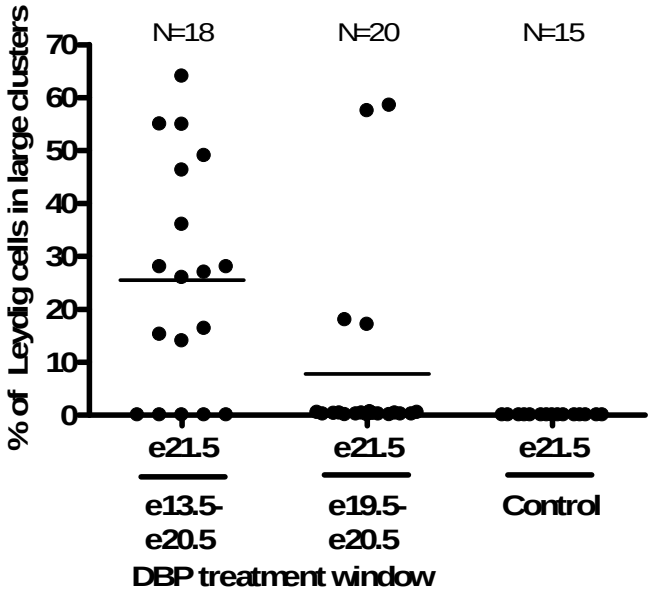

Figure 5.14 Quantification of fetal Leydig cell aggregation, and its between-animal variability, in animals exposed to DBP from e13.5-20.5, from e19.59-20.5 and in controls exposed from e13.5-20.5.

Analysis of focal dysgenetic areas in postnatal life has demonstrated that animals exposed to DBP from e19.5-20.5, who exhibit a low frequency of large Leydig cell aggregation in fetal life, do not exhibit focal areas of dysgenesis in postnatal life (Figure 5.15) suggesting that (i) exposure to DBP earlier in gestation is necessary for these to occur and (ii) major aggregation of fetal Leydig cells are a precursor to focal dysgenetic areas postnatally.

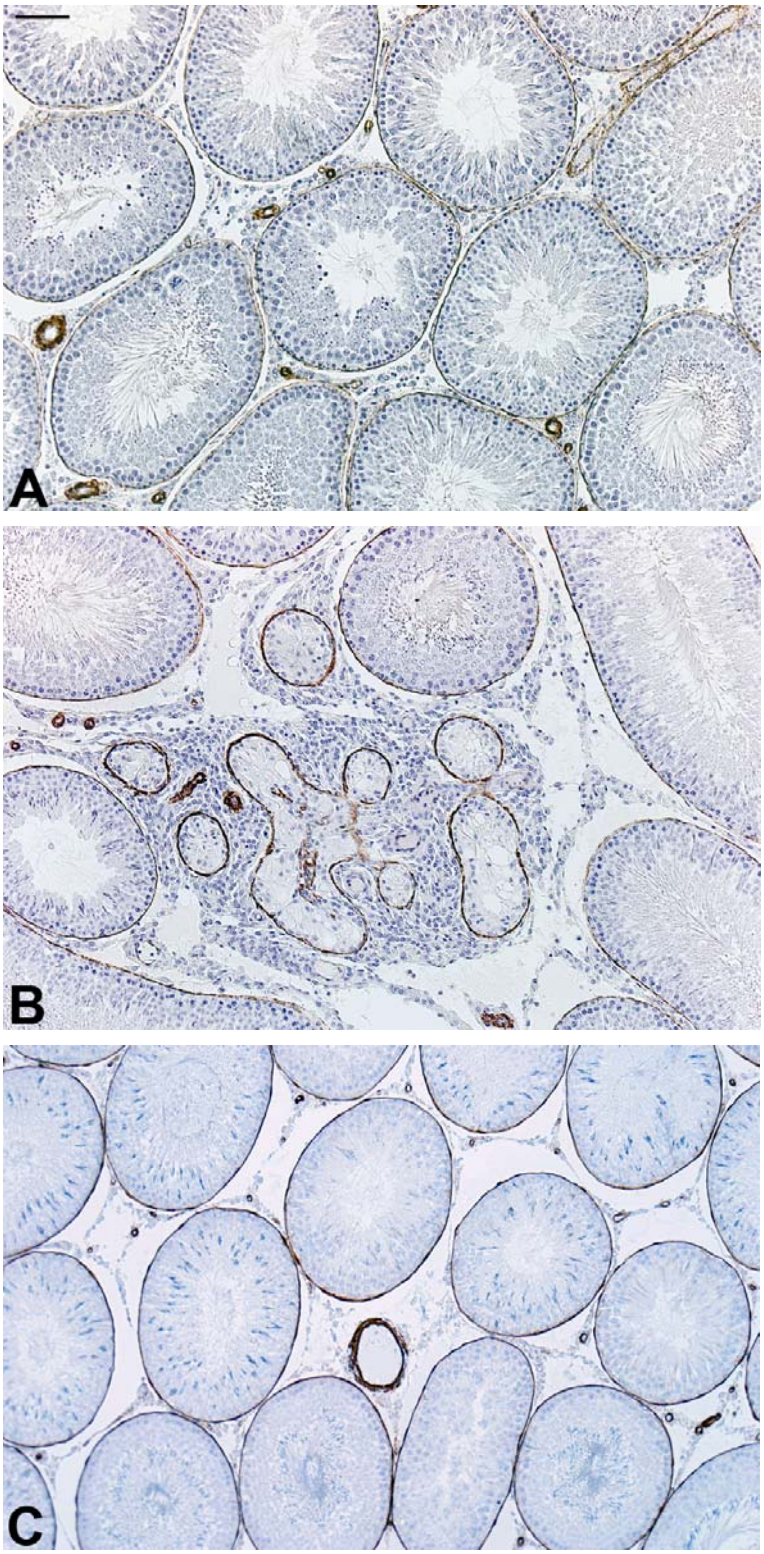

Figure 5.15 Representative photomicrographs of adult testis sections immunostained with SMA, demonstrating focal dysgenetic areas after *in utero* to DBP from e13.5-20.5 (B), but absent in control animals (A) and animals exposed in utero to DBP from e19.5-20.5 (C). Scale bar represents 100μm.

Analysis of e21.5 control testes or testes exposed to DBP from e13.5-20.5 or e11.5-20.5 immunostained with AMH revealed that some fetal Leydig cell clusters contained isolated Sertoli cells and germ cells (Figure 5.16). This has also been described in some postnatal areas of focal dysgenesis (Mahood et al., 2005). These abnormal cell 'mixtures' can also contain presumptive peritubular myoid cells (Fisher et al., 2003). This intermingling of cells did not occur in animals exposed to DBP in the late window (e19.5-20.5), demonstrating that DBP treatment from e13.5 (or e11.5) is essential for abnormal somatic cell mixes to occur.

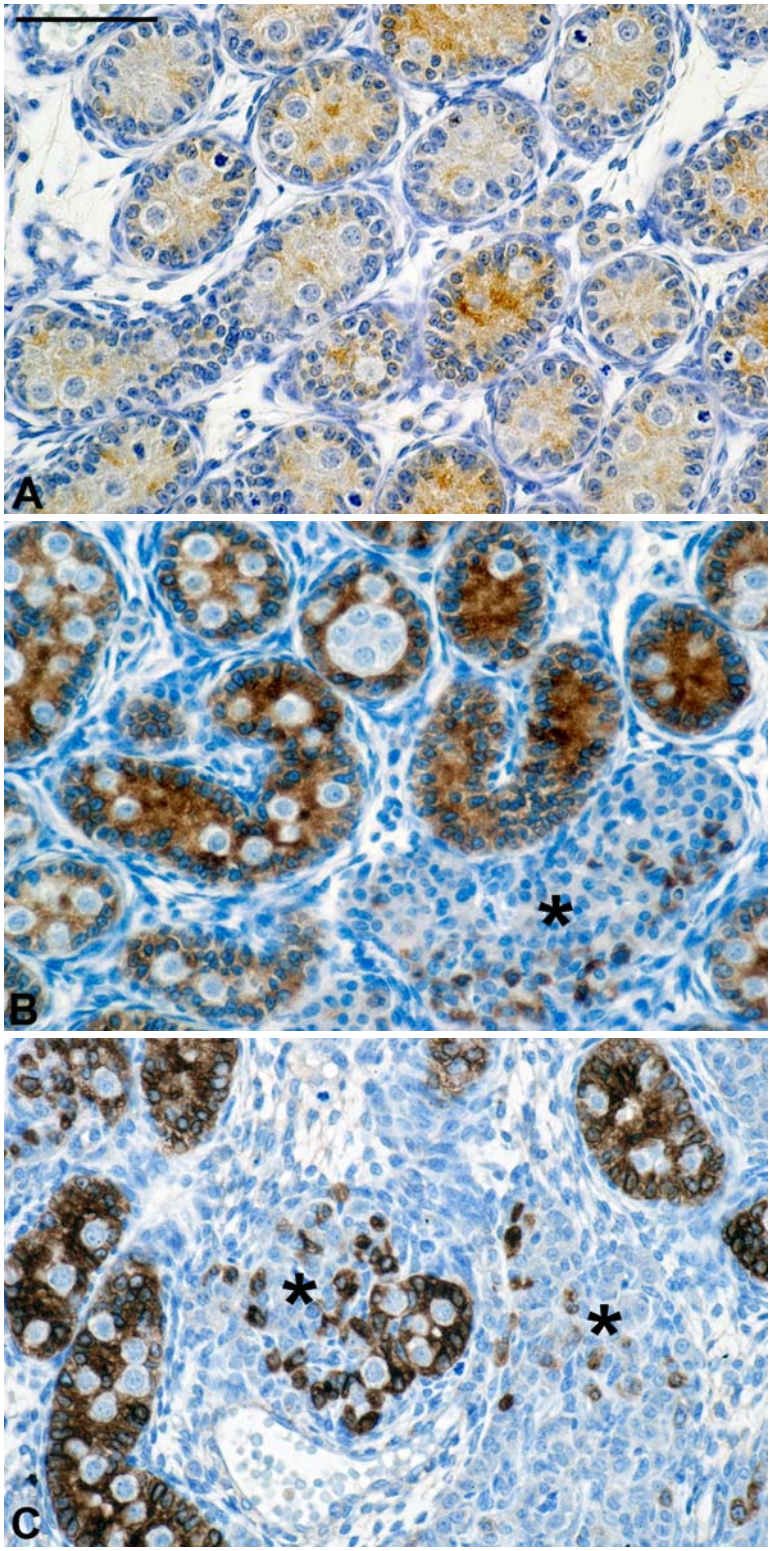

Figure 5.16 Photomicrographs depicting AMH positive Sertoli cells in the Leydig cell cluster of an e21.5 testis exposed *in utero* to DBP from e13.5-20.5 (B), Sertoli cells and germ cells in the Leydig cell cluster of an e21.5 testis exposed *in utero* to DBP from e11.5-20.5 (C), and the absence of Leydig cell clusters in the control e21.5 testis (A). Clusters are denoted by the asterisks. Scale bar represents 100μm.

5.3.6 *Tfm* and ARKO mice

The dysgenetic endpoints analysed in this chapter (occurrence of multinucleated gonocytes and Leydig cell aggregations) were also examined in e18.5 *tfm* mice, which provided a positive control, since they do not express a functional AR and consequently cannot respond to androgen action. Testes from the ARKO mice were assessed by eye, but as they are postnatal animals, and not strictly comparable, the data was not quantified. The *Tfm* mice did not exhibit an obvious increase in MNG occurrence when compared to controls (Figure 5.17).

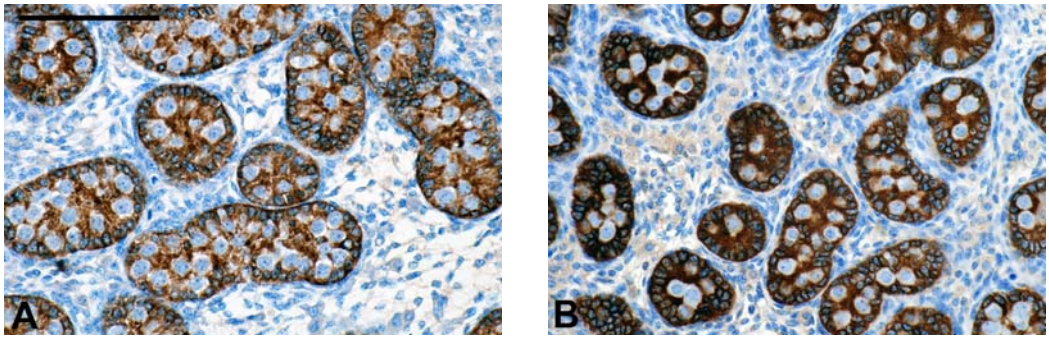

Figure 5.17 Representative photomicrographs demonstrating the absence of multinucleated gonocytes in wild type (A) and *tfm* (B) mice testes at e18.5, immunostained for AMH. Scale bar represents 100μm.

Quantification of the occurrence of multinucleated gonocytes showed that although testes from tfm mice did exhibit more multinucleated gonocytes than the wild types, this was only at a frequency of 3%, and was not statistically significant (Figure 5.18).

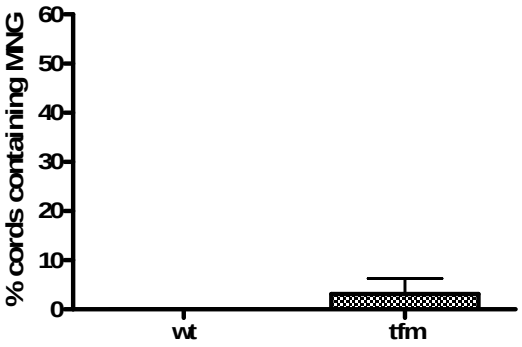

Figure 5.18 The percentage of seminiferous cords exhibiting multinucleated gonocytes at e18.5 in *tfm* (n=3) and wild type (n=3) mice. Values are means ± SEM.

Sections of ARKO and PGK-Cre testes were immunostained for AMH and assessed for multinucleated gonocytes by eye. There was no obvious increase in multinucleated gonocytes in ARKO testes compared to the PGK-Cre testes (Figure 5.19).

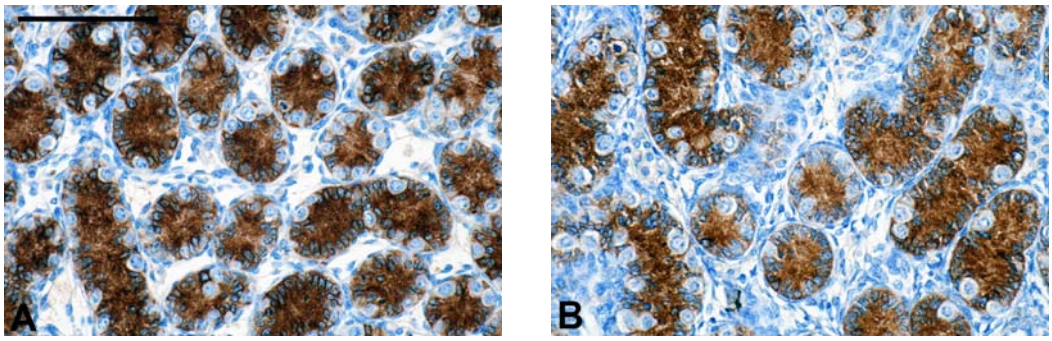

Figure 5.19 Representative photomicrographs demonstrating the absence of multinucleated gonocytes in PGK-Cre (control; A) and ARKO (B) 2 day mice testes, immunostained for AMH. Scale bar represents 100μm.

The distribution of Leydig cells in *tfm* mice at e18.5 was not obviously different from that seen in the wild types (Figure 5.20).

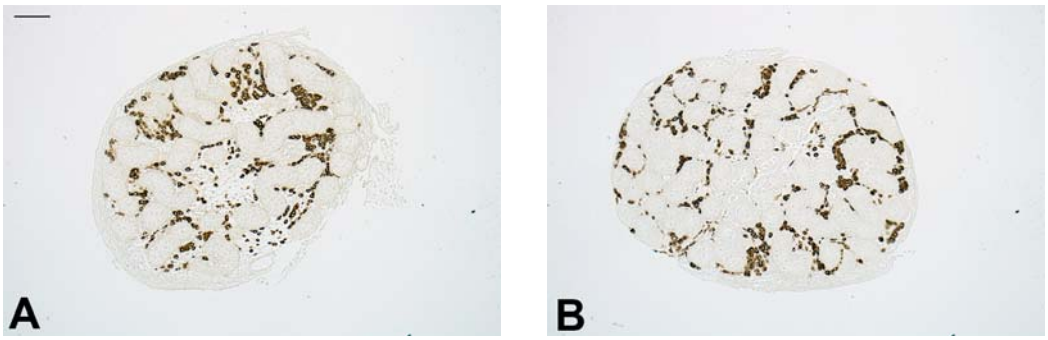

Figure 5.20 Representative photomicrographs depicting the distribution of Leydig cells (immunostained for 3β-HSD) in *tfm* (A) and wild type (B) mice at e18.5. Scale bar represents 100μm.

Quantitative Leydig cell cluster analysis confirmed that there was no difference in the distribution of Leydig cells in the *tfm* mice compared to the wild type mice (Figure 5.21).

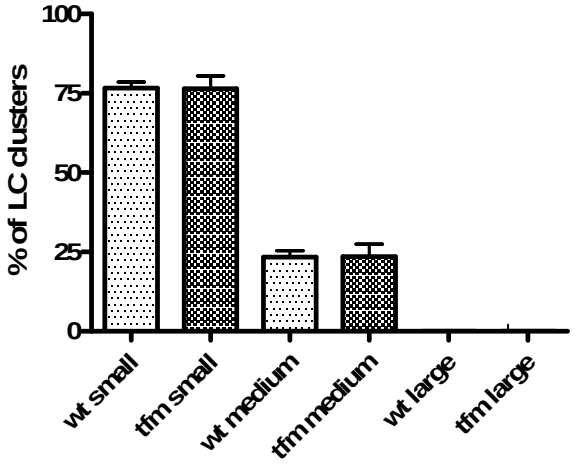

Figure 5.21 The occurrence of small, medium and large Leydig cell clusters in the testes of e18.5 *tfm* (n=3) and wild type (n=3) mice. Values are means ± SEM.

Quantitative Leydig cell analysis was not undertaken for the ARKO mice, but visual inspection of 3ß-HSD immunostained sections showed no evidence of Leydig cell aggregation at this age (Figure 5.22).

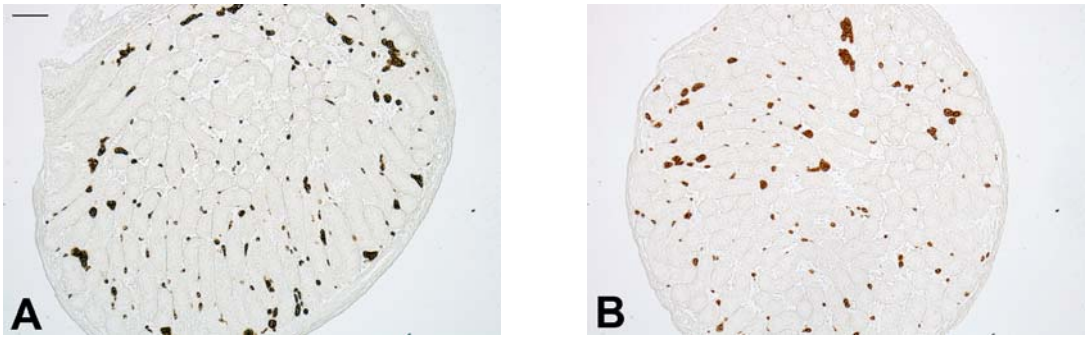

Figure 5.22 Photomicrographs demonstrating Leydig cell distribution (3β-HSD-positive, brown) in postnatal day 2 PGK-Cre (A) and ARKO (B) testes. Scale bar represents 100μm.

5.4 Discussion

The aim of this chapter was to investigate whether reduced androgen action contributes causally to the fetal dysgenetic endpoints: increased frequency of multinucleated gonocytes and Leydig cell aggregation, used as a surrogate for focal dysgenesis in postnatal life. In order to determine this, pregnant dams were exposed from e13.5-20.5 to treatments or co-treatments designed to manipulate testosterone levels (DBP, TP) or action (flutamide, DMBA), and the effect that these treatments had on testicular testosterone levels were assessed (chapter 4) and compared to the effects they also had on multinucleated gonocyte frequency and Leydig cell aggregation. The potential relationships between testicular testosterone levels and multinucleated gonocytes or Leydig cell aggregation were also investigated in animals that had been exposed to DBP during various treatment windows and assessed at e21.5 for the endpoints mentioned above. These studies were devised to ascertain whether multinucleated gonocytes and Leydig cell aggregation occurred during the treatment windows that did not reduce

testicular testosterone and therefore help clarify the role of intratesticular testosterone in multinucleated gonocyte frequency and Leydig aggregation. *Tfm* mice were also assessed to determine whether multinucleated gonocytes and/or Leydig cell aggregation occurs in the complete absence of androgen action.

As previously discussed in chapter 4, only exposure to DBP ± co-treatments, demonstrated a significant reduction in testicular testosterone. Parallel to this, only exposure to DBP ± co-treatments caused a significant increase in multinucleated gonocytes and the occurrence of large Leydig cell aggregations. Consequently, at the treatment group level, the results demonstrate a possible relationship between the suppression of intratesticular testosterone and the occurrence of multinucleated gonocytes and the formation of fetal Leydig cell clusters.

The DBP treatment window study provided similar results, with the treatment window groups shown to cause a reduction in testicular testosterone in chapter 4 (treatment from e19.5-20.5, e13.5-20.5 or e11.5-20.5), also causing an increase in multinucleated gonocyte frequency and Leydig cell aggregation. The extent to which multinucleated gonocyte frequency was increased within certain treatment windows was puzzling, however, as exposure to DBP from e19.5-20.5 resulted in the most dramatic increase in multinucleated gonocytes (52%). This was a significantly greater increase than that seen with the e13.5-20.5 treatment window (35%), which in turn was significantly greater than the increase seen after exposure from e11.5-20.5 (21%). How exposure to DBP for a shorter time period can induce a significantly greater frequency of multinucleated gonocytes is unknown, and

does not appear to be related to the extent of testicular testosterone suppression, which was broadly similar in each of these treatment groups.

Other data from our Group has shown that the occurrence of multinucleated gonocytes and Leydig cell clusters is DBP dose dependent (Mahood et al., 2007), and that a significant increase in either endpoint is only evident when the dose of DBP was sufficient to reduce testicular testosterone significantly (Mahood et al., 2007). These and the present data provide evidence that reduced intratesticular testosterone levels could be involved in the aetiology of multinucleated gonocyte formation and fetal Leydig cell aggregation, however, this evidence was not supported by parallel findings from the *tfm* and/or ARKO mice. In the absence of androgen action, these animals did not exhibit an abnormal frequency of multinucleated gonocytes or evidence for large Leydig cell clusters, which strongly suggests that, if androgen action on the fetal testis is comparable in mice and rats, these endpoints are not related causally to reduced androgen action but instead result from some other effect(s) of DBP treatment. Consistent with this conclusion, data have just been published demonstrating that *in utero* exposure of mice to DBP does not cause a reduction in testicular testosterone, but still induces formation of multinucleated gonocytes and impaired seminiferous cord formation (specifically increased cord diameter; (Gaido et al., 2007).

In this chapter, fetal Leydig cell aggregation was used as a surrogate marker for postnatal focal dysgenesis, so the relationship between these two endpoints was further investigated. Evidence from this chapter confirmed that the presence of large Leydig cell clusters is required for the formation of postnatal focal dysgenetic areas, as testes from animals exposed to DBP from e19.5-20.5 did not exhibit a high frequency of large Leydig clusters in fetal

life and did not demonstrate focal areas of dysgenesis in postnatal life. Some Leydig cell clusters in e21.5 testes from animals exposed to DBP from e13.5-20.5 or from e11.5-20.5 were also shown to contain 'out of place' Sertoli cells and germ cells. This abnormal 'mixture' of cells is also evident in some postnatal focal dysgenetic areas (Fisher et al., 2003; Mahood et al., 2005), further evidence that large fetal Leydig cell clusters leads to the postnatal formation of focal dysgenetic areas, which supports a previously published hypothesis which suggests that abnormal Leydig cell aggregation 'traps' Sertoli cells and other cell types and then, after birth, these areas attempt to form seminiferous cords and thus develop into focal dysgenetic areas (Mahood et al., 2006).

It is possible that the absence of increased multinucleated gonocytes and/or Leydig cell aggregation in response to reduced/ablated androgen action in the mouse, resulted from a species difference, and that reduced androgen action in the rat is responsible for testicular dysgenesis. This will be investigated further in chapter 6 in which, Dexamethasone, a treatment that reduces testicular testosterone, but has no known toxic effects on specific cells of the fetal testis, was administered and fetal testes from the offspring analysed for the presence of increased multinucleated gonocyte frequency and Leydig cell aggregation.

6 IUGR, intratesticular testosterone and testicular dysgenesis

6.1 Introduction

One of the common risk factors for all four disorders of the hypothesised testicular dysgenesis syndrome (TDS; cryptorchidism, hypospadias, testis cancer and low sperm counts) is intra-uterine growth restriction (IUGR) (Sharpe and Skakkebaek, 2003; Skakkebaek et al., 2001). *In utero* exposure of rats to the synthetic glucocorticoid dexamethasone has been shown to cause IUGR (Drake et al., 2005). Consequently, the aim of this chapter was to administer dexamethasone to pregnant rats, in order to investigate whether IUGR alone could induce TDS-like disorders, in association with suppression of testosterone, in the male offspring. Dexamethasone was also administered as a co-treatment with DBP (shown previously by (Barlow and Foster, 2003; Ema et al., 1998; Ema et al., 2000; Fisher et al., 2003; Mylchreest et al., 1998; Mylchreest et al., 1999; Mylchreest et al., 2000), and in chapters 4 and 5, to induce TDS-like effects in exposed male rats). DBP does not cause a significant reduction in fetal bodyweight (chapter 4), so co-treatment with dexamethasone was investigated to determine whether the induction of IUGR, in addition to DBP exposure, would exacerbate testicular dysgenesis in exposed males. However, the bodyweight of pups exposed *in utero* to dexamethasone alone in these studies was not significantly reduced in comparison to the controls (Figure 6.1), although DBP + dexamethasone did induce a significant reduction in bodyweight (Figure 6.1).

Previous publications had however, reported that prenatal exposure to dexamethasone resulted in reduced anogenital distance (AGD; (Holson et al., 1995) and reduced testosterone levels postnatally in immature (postnatal day

35) rats (Page et al., 2001).This suggested that dexamethasone could act as a useful treatment, allowing the analysis of the effects of reducing testicular testosterone without the potential direct and toxic effects of DBP on the cells of the fetal testis.

All DBP endpoints previously analysed in chapter 4 and 5 were investigated in the dexamethasone ± DBP exposed animals. Glucocorticoid receptor (GR) expression had not been specifically investigated in the fetal rat testis, raising the possibility that dexamethasone exposure caused direct effects on the cells of the fetal testis. Since it had been reported that GR mRNA is expressed in the fetal testis (Condon et al., 1998), GR protein expression was investigated in the fetal testis.

6.2 Materials and Methods

In brief, time-mated pregnant dams were treated daily using one of the regimes shown in Table 6.1.

Table 6.1 Summary of *in utero* treatment regimes used.

Treatment	Treatment window	Kill age
Control (vehicle only)	e13.5-20.5	e21.5
Dexamethasone 100µg/kg	e13.5-20.5	e21.5
DBP 500mg/kg	e13.5-20.5	e21.5
DBP 500mg/kg + Dexamethasone 100µg/kg	e13.5-20.5	e21.5

The pregnant dams were killed at e21.5 and pups removed (section 2.4). Bodyweight (section 2.4.2) and anogenital distance (section 2.4.3) were recorded before testes were removed (section 2.4.1) and weighed. After fixation (section 2.4.6), processing (section 2.4.7) and sectioning (section 2.6.1.1), testis sections were stained for WT-1 expression (section 2.6.1), and Sertoli cell number determined (sections 2.7.1). Sections of testis were also

stained with either toluidine blue (section 2.6.1.9) or for 3β-HSD expression (section 2.6.1), and the percentage of seminiferous cords containing multinucleated gonocytes determined (sections 2.7.5) or Leydig cell aggregation analysed (section 2.7.4). Sections of testis were immunostained with glucocorticoid receptor (section 2.6.1) to determine its expression in the fetal testis. Frozen testes were assayed for intratesticular testosterone levels (section 2.8.1). The number of animals used is indicated in each figure legend. Animals came from a minimum of three litters in all cases.

6.2.1 Statistical analysis

All data were analysed using one-way ANOVA followed by the Bonferroni post test.

6.3 Results

Fetal bodyweight was expected to decrease in animals treated *in utero* with dexamethasone, however, no significant decrease was observed, perhaps as a result of low 'n' numbers. DBP exposure did not cause a significant change in bodyweight either, but when the two treatments were combined, a 24% reduction in bodyweight was induced (Figure 6.1).

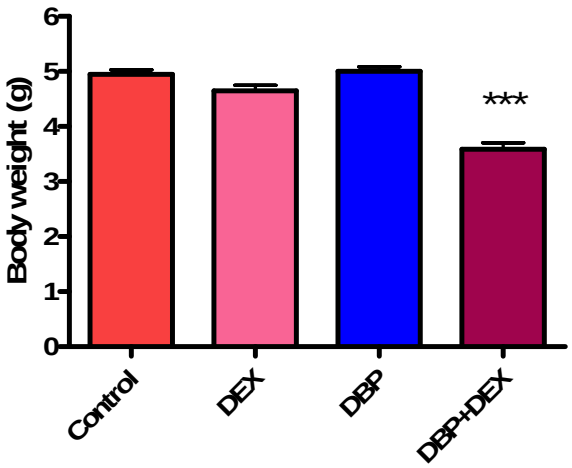

Figure 6.1 Effect of *in utero* exposure to dexamethasone (n=7), DBP (n=13), DBP + dexamethasone (n=6) or vehicle alone (control; n=17) on the bodyweights of fetuses at e21.5. Values are means ± SEM. ***p<0.001 in comparison to control value.

Exposure to DBP resulted in a 31% decrease in testis weight compared to control animals (Figure 6.2). Dexamethasone exposure on its own did not induce a reduction in testis weight, but when combined with DBP treatment, resulted in a 45% reduction in testis weight, which was greater than the reduction in animals exposed to DBP alone (Figure 6.2).

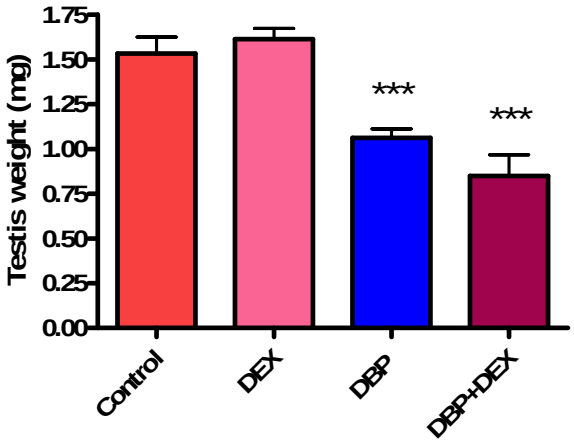

Figure 6.2 Effect of *in utero* exposure to dexamethasone (n=7), DBP (n=16), DBP + dexamethasone (n=6) or vehicle alone (control; n=15) on testis weight in e21.5 rats. Values are means ± SEM. ***p<0.001 in comparison to control value.

6.3.1 Testicular testosterone levels

Testicular testosterone levels were significantly reduced in treatment groups. Animals exposed to dexamethasone demonstrated a 53% reduction, those exposed to DBP exhibited a 54% reduction and those exposed to DBP + dexamethasone demonstrated a 75% reduction in intratesticular testosterone levels (Figure 6.3).

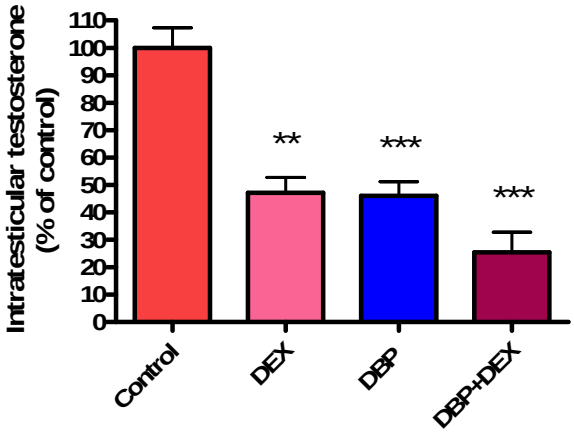

Figure 6.3 Intratesticular testosterone levels at e21.5 in male rats exposed *in utero* to dexamethasone (n=4), DBP (n=5), DBP + dexamethasone (n=5) or vehicle alone (control; n=11). Testicular extracts were not all assayed together. To avoid consequent inter-assay variation, testosterone levels for testes from treated animals were expressed as a percentage of the mean value for control testes run in the same assay. **p<0.01, ***p<0.001 in comparison to control value.

6.3.2 Anogenital distance (AGD)

Male pups exposed to dexamethasone and DBP showed similar reductions in AGD (18% and 23% respectively), however, the reduction seen after dexamethasone exposure was not statistically significant due to high between-animal variation. Neither of these two treatment groups reduced AGD to values comparable with female controls. When DBP and dexamethasone were administered together, a greater reduction in fetal AGD (42%) was observed in comparison to that observed in males exposed to either treatment alone (Figure 6.4).

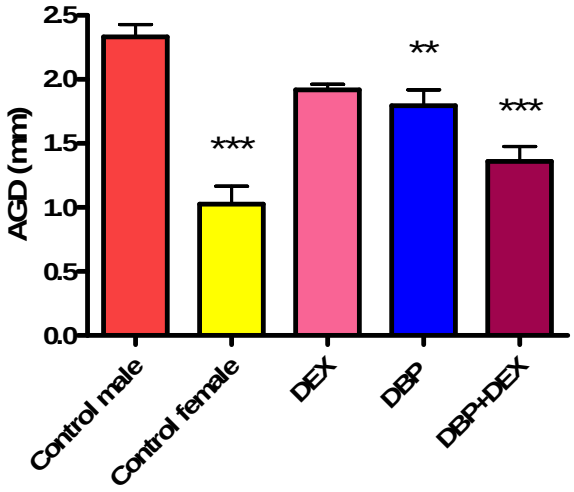

Figure 6.4 Anogenital distance at e21.5 in male rats exposed *in utero* to dexamethasone (n=7), DBP (n=10), DBP + dexamethasone (n=6) or vehicle alone (control; male n=12 and female n=8). Values are means ± SEM. **p<0.01, ***p<0.001 in comparison to control male value.

6.3.3 Sertoli cell number

Sertoli cell number per testis was reduced by 48% in animals exposed to dexamethasone alone or DBP alone (Figure 6.5). Animals exposed to DBP and dexamethasone combined, demonstrated a 66% reduction in Sertoli cell number per testis (Figure 6.5).

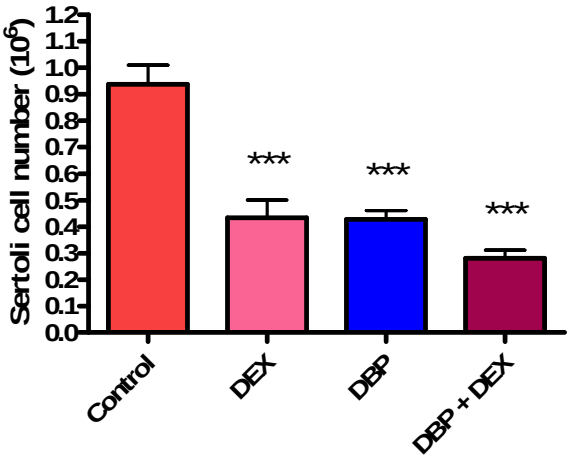

Figure 6.5 Effect of *in utero* exposure to dexamethasone (n=5), DBP (n=10), DBP + dexamethasone (n=6) or vehicle alone (control; n=12) on Sertoli cell number in e21.5 rat testes. Values are means ± SEM. ***p<0.001 in comparison to control value.

6.3.4 Occurrence of multinucleated gonocytes

Sections of testis from e21.5 rats exposed to dexamethasone, DBP, DBP + dexamethasone or vehicle alone (control), were counterstained with toluidine blue. This allowed the detection and enumeration of any multinucleated gonocytes present within the seminiferous cords. Multinucleated gonocytes were identified in all treatment groups, including the control group, although they were far more common in animals exposed *in utero* to DBP, either alone or combination with dexamethasone (Figure 6.6).

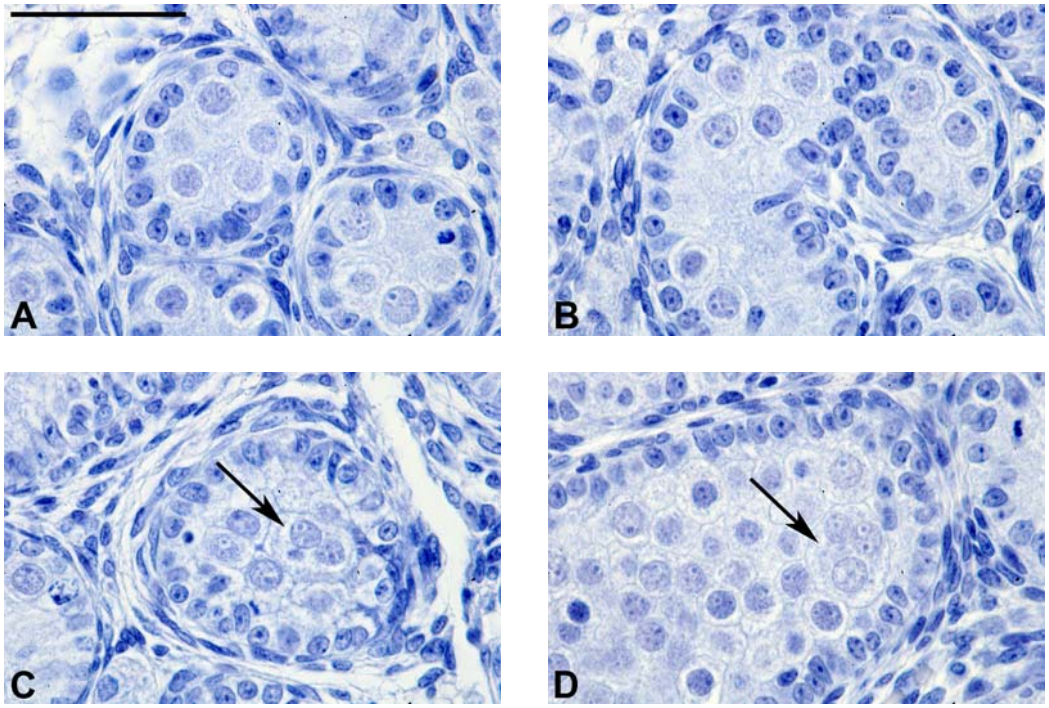

Figure 6.6 Photomicrograph demonstrating the occurrence of multinucleated gonocytes (arrows) in e21.5 male rats exposed to (A) vehicle alone (control), (B) dexamethasone, (C) DBP or (D) DBP + dexamethasone. Sections of testis were stained with haematoxylin. Note that only in animals exposed to DBP is there abnormal aggregation of gonocytes in the centre of the seminiferous cords. Scale bare represents 50μm.

In utero exposure to DBP resulted in a 44-fold increase in the percentage of cords exhibiting multinucleated gonocytes, and exposure to DBP and Dexamethasone combined, resulted in a 26-fold increase in the percentage of cords containing multinucleated gonocytes (Figure 6.7). Exposure to dexamethasone alone however, had no effect on the prevalence of multinucleated gonocytes within the fetal testis, when compared with controls (Figure 6.7).

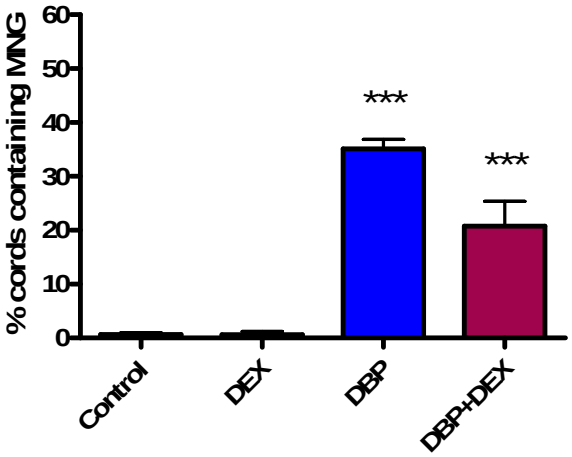

Figure 6.7 The percentage of seminiferous cords exhibiting multinucleated gonocytes (MNG) at e21.5 after *in utero* exposure to dexamethasone (n=6), DBP (n=10), DBP + dexamethasone (n=6) or vehicle alone (control; n=12). Values are means ± SEM. ***p<0.001 in comparison to control value.

6.3.5 Leydig cell clusters

Changes in Leydig cell distribution were clearly visible in the DBP alone and DBP + dexamethasone treatment groups, when compared to controls (Figure 6.8). In these animals abnormally large Leydig cell clusters were present in the centre of the testes and there was a corresponding decrease in the number of small Leydig cell clusters. Leydig cell distribution in testes from animals exposed to dexamethasone alone was not obviously different from control sections (Figure 6.8).

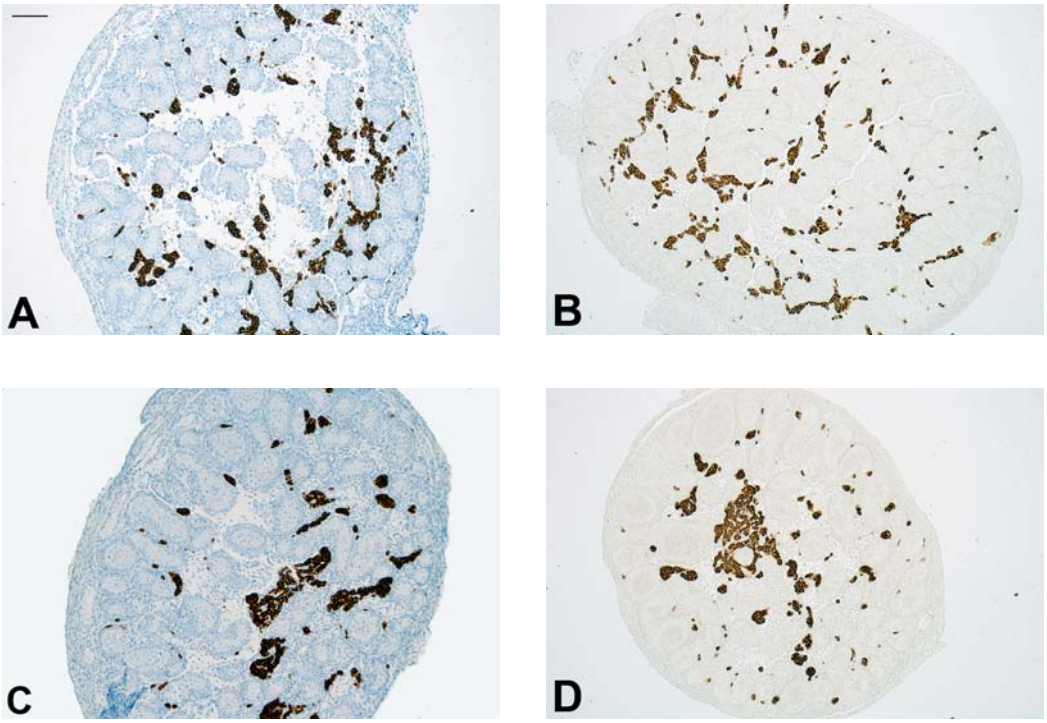

Figure 6.8 Representative photomicrographs demonstrating the distribution of Leydig cells (immunostained for 3β-HSD) in e21.5 rat testes exposed to (A) vehicle only (control), (B) dexamethasone, (C) DBP or (D) DBP + dexamethasone. Exposure to DBP or DBP + dexamethasone caused a change in Leydig cell distribution, reducing the number of small Leydig cell clusters and increasing the number of medium and large Leydig cell clusters. Scale bar represents 100μm.

Leydig cell aggregation was quantified by cluster analysis and revealed a significant reduction in the percentage of total Leydig cell cluster area accounted for by small Leydig cell clusters in animals exposed to DBP alone or DBP combined with dexamethasone (Figure 6.9). Exposure to dexamethasone alone did not cause a significant reduction in small Leydig cell clusters (Figure 6.9).

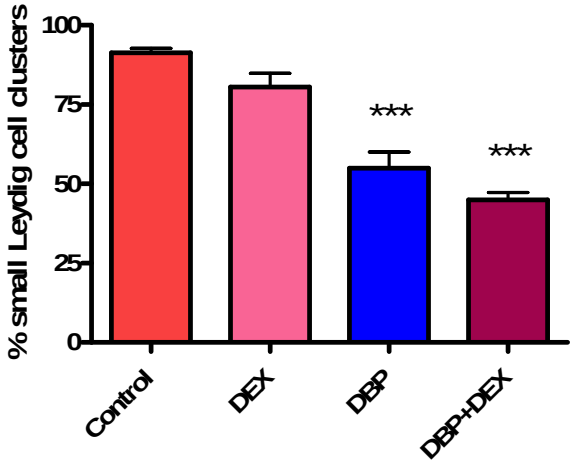

Figure 6.9 The occurrence of small size Leydig cell clusters in the testes of e21.5 rats exposed *in utero* to corn oil (control; n=10), dexamethasone (n=6), DBP (n=6) or DBP + dexamethasone (n=6). Values are means ± SEM. ***p<0.001 in comparison to control value.

Medium sized Leydig cell clusters were evident in all treatment groups, including the control, but were significantly increased in DBP alone and DBP + dexamethasone exposed animals (Figure 6.10).

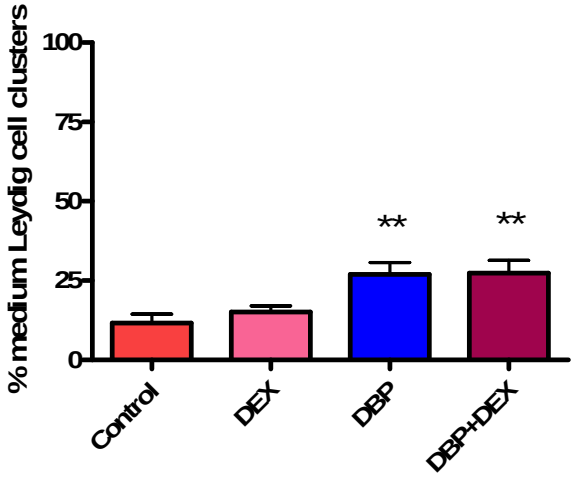

Figure 6.10 The occurrence of medium size Leydig cell clusters in the testes of e21.5 rats exposed *in utero* to corn oil (control; n=10), dexamethasone (n=6), DBP (n=6) or DBP + dexamethasone (n=6). Values are means ± SEM. **p<0.01 in comparison to control value.

Large Leydig cell clusters were detectable in all treatment groups, but at very low levels in controls (0.5%) and dexamethasone exposed animals (4.3%). The percentage of total Leydig cell cluster area accounted for by large Leydig cell clusters was significantly increased in animals exposed to DBP alone or DBP + dexamethasone (Figure 6.11).

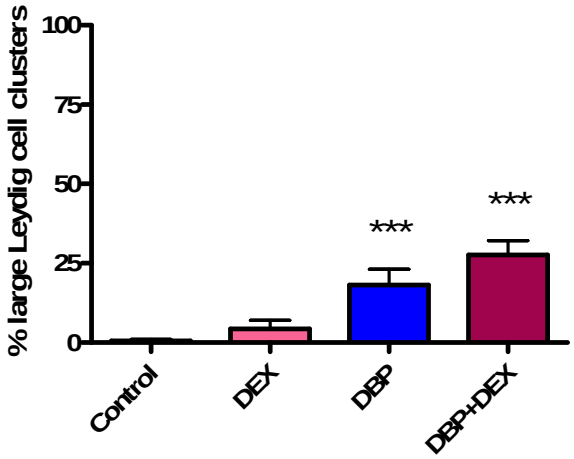

Figure 6.11 The occurrence of large size Leydig cell clusters in the testes of e21.5 rats exposed *in utero* to corn oil (control; n=10), dexamethasone (n=6), DBP (n=6) or DBP + dexamethasone (n=6). Values are means ± SEM. ***p<0.001 in comparison to control value.

6.3.6 Glucocorticoid receptor expression

Although a parallel reduction in testicular testosterone levels and Sertoli cell number was found at e21.5 after dexamethasone exposure, consistent with androgen regulation of Sertoli cell proliferation, it became evident that other possible mechanisms for the reduction in Sertoli cell number would have to be considered. For example, it was possible that dexamethasone might have a direct effect on the Sertoli cells, since one publication had suggested that the GR was expressed in fetal the testis (Condon et al., 1998). Immunohistochemical analysis of GR expression in the fetal testis demonstrated that at e17.5 the Sertoli cells were generally immunonegative, although occasional cells were positive and the nucleoli of the Sertoli cells

and germ cells appeared to be immunostained. At this age, the peritubular myoid cells and interstitial cells were uniformly immunopositive. At e21.5 the Sertoli cells were predominantly immunopositive, and the peritubular myoid cells and interstitial cells also remained strongly immunopositive (Figure 6.12).

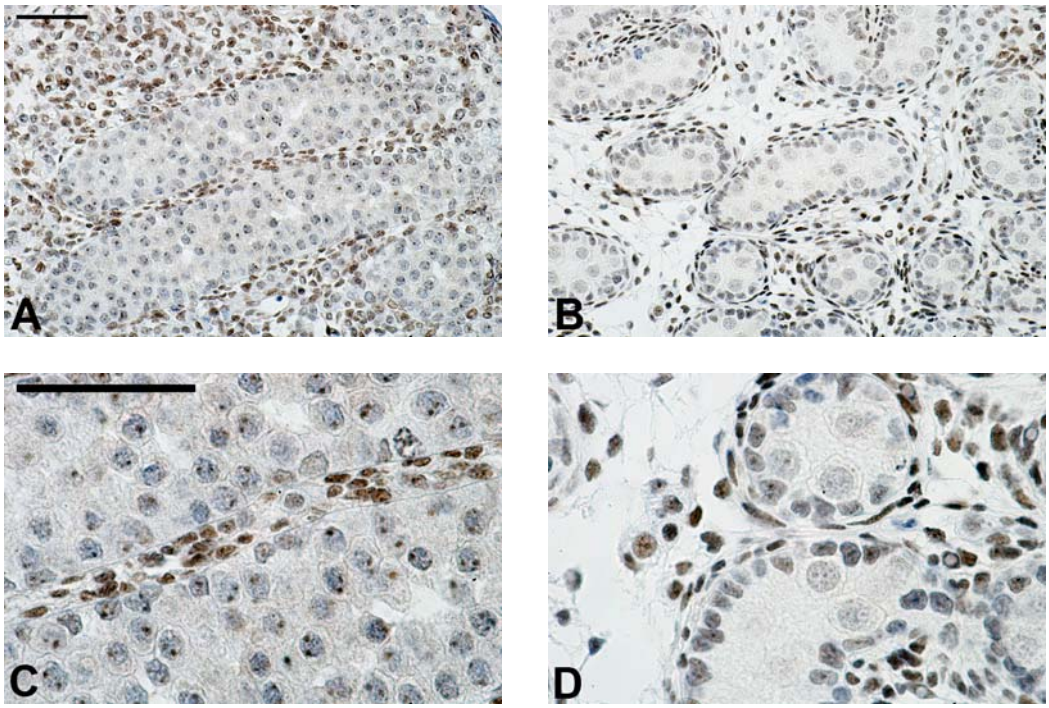

Figure 6.12 Glucocorticoid receptor immunoexpression at (A & C) e17.5 and (B & D) e21.5 in control fetal rat testes. Scale bars represent 50μm.

6.4 Discussion

This chapter sought to determine whether dexamethasone-induced IUGR would result in TDS-like disorders in rats exposed *in utero*, since IUGR is one of the main risk factors for TDS (Sharpe and Skakkebaek, 2003; Skakkebaek et al., 2001), and to investigate whether *in utero* exposure to dexamethasone causes a reduction in testicular testosterone levels. It also sought to determine whether co-treatment of dexamethasone with DBP would exacerbate DBP-induced testicular dysgenetic endpoints. Dexamethasone

exposure, however, did not induce a significant reduction in bodyweight. It is possible that this was due to low 'n' numbers, since this study consisted of bodyweights from seven animals, from five litters, whereas a previous publication which reported a significant reduction ($p<0.05$) in bodyweight, had an 'n' of 74 (Drake et al., 2005).

Despite failing to cause IUGR, dexamethasone significantly reduced testicular testosterone and reduced AGD, although this reduction was not significant, possibly due to high between-animal variation. These findings confirm previous reports that prenatal exposure to dexamethasone causes a reduction in AGD (Holson et al., 1995) and suppressed testiscular testosterone levels in postnatal rats (Page et al., 2001). This meant that Sertoli cell number, the occurrence of multinucleated gonocytes and Leydig cell aggregation could be analysed in fetal rats with reduced testicular testosterone, but without the potential direct toxic effects of DBP.

Sertoli cell number was significantly reduced in animals exposed *in utero* to dexamethasone alone and when co-administered with DBP. The magnitude of reduction in Sertoli cell number with dexamethasone ± DBP was almost identical to that found for testicular testosterone levels. Exposure to dexamethasone + DBP resulted in a slightly greater reduction in testicular testosterone and Sertoli cell number than did exposure to either dexamethasone or DBP alone, but this was not significantly different. Once again, these results are consistent with androgens playing a role in promoting Sertoli cell proliferation/number.

Increased frequency of multinucleated gonocytes and Leydig cell aggregation were not induced by dexamethasone treatment alone. These endpoints were only affected after DBP ± dexamethasone exposure,

strengthening the findings from chapter 5, which suggested that these endpoints of testicular dysgenesis do not result from reduced androgen action, but are the result of other effects of DBP exposure. Moreover, despite causing a significant reduction in bodyweight, co-treatment with dexamethasone + DBP did not exacerbate these dysgenetic endpoints; in fact, fewer (14%) cords exhibited multinucleated gonocytes after co-treatment than after DBP treatment alone and co-treatment only resulted in a slightly higher prevalence large Leydig cell clusters than did treatment with DBP alone. This suggests that reduction in bodyweight may not be as important for these TDS-like endpoints as for other TDS disorders.

The effects of IUGR on the induction of the TDS-like endpoints investigated in this thesis were also analysed in rats that had been exposed *in utero* to testosterone propionate (TP), as seen in chapters 4 and 5. Although TP was administered in these studies to ascertain which, if any, of the DBP effects could be prevented if the DBP-induced reduction in intratesticular testosterone levels was attenuated, *in utero* exposure to TP has also been shown to cause IUGR (Fritz et al., 1984; Manikkam et al., 2004). However, although *in utero* TP exposure did result in a significant reduction in the bodyweight of the offspring, there was no consistent relationship between reduced bodyweight and Sertoli cell number (chapter 4), although this was accounted for by a concurrent reduction in testicular testosterone levels and, as with dexamethasone treatment, TP exposure did not induce multinucleated gonocytes or Leydig cell aggregation (chapter 5), despite having reduced testicular testosterone levels. These results suggest that reduced bodyweight does not contribute to the TDS-like endpoints investigated in this thesis.

The mechanism by which testicular testosterone is reduced in animals exposed to dexamethasone is unknown, although several publications point to a possible mechanism. Dexamethasone exposure in adult rats has been reported to cause an increase in endogenous DAX1 (dosage sensitive sex reversal adrenal hyperplasia congenital critical region on the X chromosome, gene 1) expression (Gummow et al., 2006; Yu and Li, 2006). This increase in DAX1 results in increased amounts of complexed DAX1:Sf1 (steroidogenic factor 1), so that although dexamethasone exposure does not alter the expression of Sf1 itself, it causes a reduction in free Sf1 (Gummow et al., 2006; Yu and Li, 2006). Sf1 regulates the expression of StAR (steroidogenic acute regulatory protein), a transport protein that regulates cholesterol transfer from the outer mitochondrial membrane to the inner membrane, where cytochrome $P450_{scc}$ enzyme cleaves the cholesterol side chain in the first step of steroidogenesis, making this a rate-limiting step in steroidogenesis. This suggests that dexamethasone may suppress testicular testosterone production by increasing the amount of complexed DAX1:Sf1, resulting in a reduction of free Sf1 and a concordant reduction in StAR expression (Yu and Li, 2006). There is however, still some debate and uncertainty as to where DAX1 is expressed in the fetal testis, and in order for this mechanism to be viable, it would need to be expressed in the fetal Leydig cells. One publication reported DAX1 expression in the Leydig cell cytoplasm of fetal rat testes (Majdic and Saunders, 1996), although the authors did comment that this result was surprising since DAX1 was thought to be a transcription factor and was therefore expected to be expressed in the Leydig cell nucleus. Another publication used *in situ* analysis of DAX1 RNA in mouse embryos to demonstrate expression in the interstitium from e14.5 to e17.5, and suggested that this was restricted to the fetal Leydig cells (Ikeda et al., 1996), while

another reported DAX1 expression in the Sertoli cells and other somatic cells immediately beneath the coelomic epithelium in fetal mice (Meeks et al., 2003). Immunohistochemistry using a DAX1 antibody was performed for the present study, but was unsuccessful. However, DAX1 mRNA expression levels were measured in control and DBP exposed fetal testes in chapter 7, and DAX1 mRNA expression was in fact, reduced in DBP exposed testes, which also present with reduced testicular testosterone levels. When DAX1 mRNA expression was corrected for Sertoli cell number, this DBP-induced reduction was no longer evident, suggesting that DAX1 mRNA is expressed in Sertoli cells in fetal rat testes. Consequently it is not possible to confirm this potential mechanism for testicular testosterone reduction with dexamethasone exposure.

Results from this chapter reveal that dexamethasone exposure results in a reduction in testicular testosterone and a concurrent reduction in Sertoli cell number, and exposure to dexamethasone + DBP causes a greater reduction in testicular testosterone and Sertoli cell number. However, immunohistochemical analyses of GR expression in the fetal testis has demonstrated that the GR is expressed in only a few Sertoli cells at e17.5 and in most Sertoli cells at e21.5. This proposed GR expression pattern, suggesting that GR switches on in Sertoli cells towards the end of fetal life, fits with a previous publication describing immature Sertoli cells in culture, that expressed GR and were stimulated by glucocorticoids (Levy et al., 1989). As a consequence of this expression, it is not possible to unequivocally state that the reduction in Sertoli cell number seen after *in utero* exposure to dexamethasone is due to the reduction in testicular testosterone and not through the direct actions of dexamethasone on the Sertoli cell.

As with DBP, if the dexamethasone-induced reduction in Sertoli cell number is the result of reduced testicular testosterone, then androgen action must be mediated via another cell type, as discussed in chapter 4, as fetal Sertoli cells do not express AR. It has been hypothesised that the peritubular myoid cell may mediate androgen action on the fetal Sertoli cell and this will be investigated further in chapter 7.

7 Mediators of androgen action or dysgenesis

7.1 Introduction

Earlier studies in this thesis have suggested that androgens play a role in fetal Sertoli cell proliferation (chapters 3 and 4). However, as discussed previously in chapter 3, Sertoli cells do not express androgen receptors (AR) in fetal life (Williams et al., 2001) and chapter 3). Consequently, it has been hypothesised that androgen action on the fetal Sertoli cells is mediated via the peritubular myoid cells. The peritubular myoid cells do express AR during fetal life (Sharpe, 2005; Tan et al., 2005) and chapter 3) and have been shown previously to communicate with the Sertoli cells, in culture (Buzzard et al., 2003a; Skinner and Fritz, 1985b) and during cord formation, when both cell types contribute to the formation of the basement membrane (Skinner et al., 1985; Tung, 1984).

In utero exposure to DBP was shown in chapter 4 to reduce testicular testosterone by 48% and Sertoli cell number by 49% in male offspring at e21.5. Testes from DBP-exposed animals were therefore used in this chapter to investigate the role that the peritubular myoid cells may play in mediating androgen action on the fetal Sertoli cells. Immunohistochemical markers of the peritubular myoid cell, peritubular myoid precursor cells or components of the basement membrane (laid down by both the peritubular myoid cells and Sertoli cells), were explored at e15.5, e17.5, e19.5 and e21.5, to determine whether reduced testicular testosterone or DBP exposure caused a change or delay of expression, indicating a change in peritubular myoid cell morphology or function. Markers of Sertoli cell intermediate filament proteins were also investigated to determine whether reduced testosterone or DBP exposure altered the structure of the Sertoli cells. Due to time restriction,

the investigations of some Sertoli cell markers are preliminary and were limited to control and DBP exposed testes at e21.5 only. In some cases peritubular myoid cell markers were also investigated in testes from ARKO mice, to determine whether, in the complete absence of androgen action, peritubular myoid cell morphology or function changes. However, due to time restrictions and because some of these markers did not work in the mouse, only SMA expression was thoroughly investigated. The immunohistochemical markers investigated and their known or suspected sites of expression are listed in Table 7.1.

Table 7.1 Table of potential DBP-sensitive peritubular myoid or Sertoli cell markers.

Candidate marker	Known/suspected site of expression
Androgen receptor (AR)	Peritubular myoid cell
Desmin	Intermediate filament protein (IFM) of contractile (peritubular myoid) cell
Laminin	Extracellular matrix
Nestin	IFM of Sertoli cell
Pancytokeratin	IFM of epithelial (Sertoli) cell
Periodic acid schiff	Proteoglycans of basal lamina
P75 neurotrophin receptor (P75NTR)	Peritubular myoid precursor cell
Smooth muscle actin (SMA)	Mesenchymal (peritubular myoid) cell
Vimentin	IFM of Sertoli cell

Results from chapter 3 also suggested that androgens may be involved in the regulation of peritubular myoid cell proliferation, as peritubular myoid cell numbers increased in parallel with the fetal increase in testicular testosterone levels. Peritubular myoid cell number was therefore investigated at e17.5 and e21.5 after DBP exposure.

In addition to investigating the peritubular myoid cells as potential mediators of androgen action on the Sertoli cell, non-androgen related

mechanisms were also investigated. A number of genes known or suspected to be involved in fetal testis development, and/or implicated in testicular dysgenesis in knockout studies, were investigated at various fetal ages (e15.5, e17.5 and e21.5) in testes from control and DBP exposed animals, to identify any other possible mechanisms by which DBP could cause the effects investigated in this thesis, i.e. reduced Sertoli cell number and testicular dysgenesis. Genes were investigated using Taqman quantitative RT-PCR, and quantitative mRNA expression levels at e15.5, e17.5 and e21.5, in testes from control and DBP-exposed animals, were established. The genes analysed, their known or suspected sites of expression and known or suspected roles in fetal testis development are listed in Table 7.2.

Table 7.2 Table of candidate genes.

Gene	Known/suspected site of expression	Known/suspected role in fetal testis development
DAX1	Fetal mice Sertoli cells (Meeks et al., 2003), fetal rat Leydig cell cytoplasm (Majdic and Saunders, 1996), fetal mice Leydig cells (Ikeda et al., 1996)	DAX1 deficient mice have clusters of poorly differentiated Sertoli cells, Leydig cell hyperplasia (Jeffs et al., 2001), reduced peritubular myoid cells & incomplete cord formation (Meeks et al., 2003)
Dhh	Fetal mice Sertoli cells (Bitgood and McMahon, 1995; Bitgood et al., 1996)	Inhibition of Dhh signalling leads to disrupted cords (Yao and Capel, 2002), incomplete Leydig cell differentiation (Yao et al., 2002) & mice lacking Dhh are under-masculinised (Clark et al., 2000)
DMRT1	Fetal mice Sertoli cells and germ cells (Raymond et al., 1999)	Elimination of DMRT1 does not affect embryonic gonadogenesis, but in postnatal testes germ cells are lost (Raymond et al., 2000) & Sertoli cells do not mature (Kim et al., 2007)
FGF9	Fetal mice Sertoli cells (Kim et al., 2006)	Cell proliferation, mesonephric cell migration, testis cord formation & Sertoli cell differentitation (Colvin et al., 2001)
FMR1	Human fetal primordial germ cells (Tamanini et al., 1997) and expected to be in Sertoli cells (Sharpe et al., 2003)but not so far reported	Inactivation of FMR1 leads to increased Sertoli cell proliferation (Slegtenhorst-Eegdeman et al., 1998)

7.2 Materials and Methods

In brief, time-mated pregnant dams were treated daily using one of the regimes shown in Table 7.3.

Table 7.3 Summary of *in utero* treatment regimes used.

Treatment	Treatment window	Kill age
Control (vehicle only)	e13.5-14.5	e15.5
Control (vehicle only)	e13.5-16.5	e17.5
Control (vehicle only)	e13.5-18.5	e19.5
Control (vehicle only)	e13.5-20.5	e21.5
DBP 500mg/kg	e13.5-14.5	e15.5
DBP 500mg/kg	e13.5-16.5	e17.5
DBP 500mg/kg	e13.5-18.5	e19.5
DBP 500mg/kg	e13.5-20.5	e21.5
Flutamide 100mg/kg	e13.5-20.5	e21.5
DMBA 400µg/kg	e13.5-20.5	e21.5

The pregnant dams were killed at e15.5, e17.5, e19.5 or e21.5 and pups removed (section 2.4). After fixation (section 2.4.6), processing (section 2.4.7) and sectioning (section 2.6.1.1), testes were stained for AR expression (section 2.6.1), and peritubular myoid cell number determined (sections 2.7.1) at e17.5 and e21.5. Sections from e15.5, e17.5, e19.5 and e21.5 were also stained for various markers described above in Table 7.1. Immunohistochemistry for SMA was also performed on day 2 testes from ARKO, PGK-Cre mice and e21.5 testes from flutamide and DMBA exposed animals.

Quantification of SMA expression was performed using Image-Pro Plus 4.5.1 software, and compared the intensity of SMA staining in the peritubular myoid cells to that in an internal control, blood vessels (section 2.7.6).

Frozen testes from e15.5, e17.5 and e21.5 rats were homogenised and RNA extraction performed (section 2.10.2). cDNA was synthesised from the RNA

(section 2.10.3) and Taqman quantitative RT-PCR performed, using primers (section 2.10.1) for the genes listed in Table 7.2.

Sections of e15.5, e17.5, e19.5 and e21.5 testes from control and DBP treated animals were immunostained for DMRT1 to determine whether expression in the rat fetal testis is comparable to that in the mouse and/or affected by DBP exposure.

Although mRNA levels were corrected for total 18S ribosomal RNA, which may correct to some extent any change in cell number, this wouldn't correct for a relative change in a particular cell number, for example the Sertoli cell. Consequently major changes in Sertoli cell number in DBP-exposed animals were corrected for if the gene was expected or reported to be expressed in the fetal Sertoli cell. Leydig cell, peritubular myoid cell, interstial cell numbers did not change and germ cell number only changed marginally. Correction were performed by dividing each individual Taqman sample result by the mean Sertoli cell number for that age and treatment group as cell number and mRNA levels were not detected in the same testes or the same animals.

The number of animals used for analyses in this chapter is indicated in each figure legend. Animals came from a minimum of three litters in all cases.

7.3 Results

7.3.1 Peritubular myoid cell number

Peritubular myoid cell number was not significantly changed in animals exposed to DBP 500mg/kg from e13.5 and killed on e17.5 or on e21.5 (Figure 7.1). Peritubular myoid cell number increased 11-fold in control and 8-fold in DBP-exposed animals between e17.5 and e21.5.

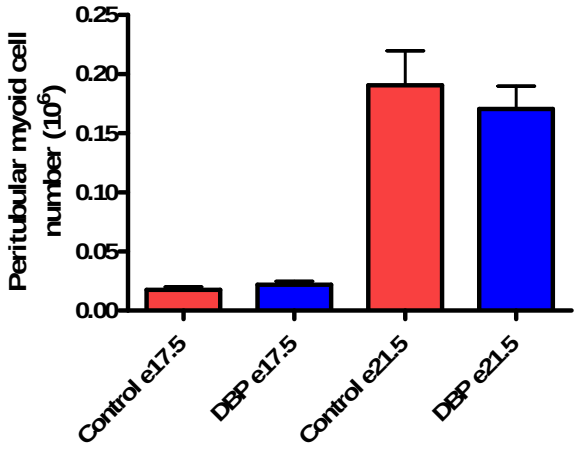

Figure 7.1 Peritubular myoid cell numbers from control (e17.5 n=5 and e21.5 n=6) and DBP-exposed (e17.5 n=5 and e21.5 n=6) animals, treated from e13.5 to the day prior to kill. Values are means ± SEM.

7.3.2 Sertoli cell/peritubular myoid cell ratio

The ratio of Sertoli cells (mean value) to peritubular myoid cells at e17.5 and e21.5 in control and DBP-exposed animals was investigated and revealed that in the control animals the average number of Sertoli cells per peritubular myoid cell decreased from e17.5 to e21.5, reflecting the greater increase in peritubular myoid cell number (11 fold), compared to the increase seen in Sertoli cell number (7.5 fold). However, in the DBP treated animals, the average number of Sertoli cells per peritubular myoid cells was significantly reduced at e17.5 when compared to the control and did not change between e17.5 and e21.5, where it was still reduced compared to the e21.5 control, but not significantly so . This reflects the consistent level of reduction in Sertoli cell number at e17.5 (52%) and e21.5 (49%), as reported in chapter 4, while the peritubular myoid cells proliferated as normal (Figure 7.2).

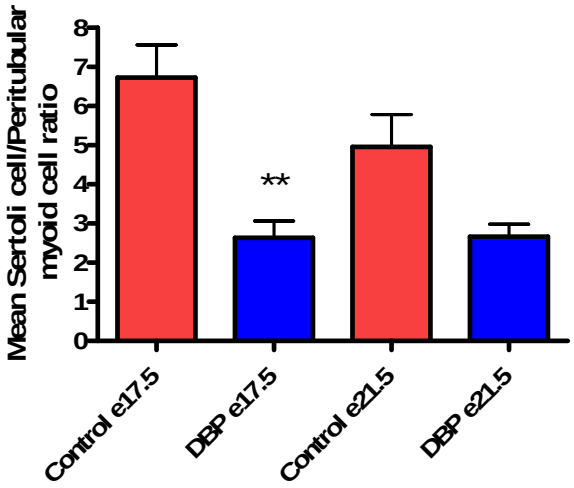

Figure 7.2 Mean Sertoli cell to peritubular myoid cell ratio in e17.5 (control n=5, DBP n=5) and e21.5 (control n=6, DBP n=6) fetal rat testes.

7.3.3 Potential DBP-sensitive peritubular myoid cell or Sertoli cell markers

7.3.3.1 Peritubular myoid cell marker

AR expression was analysed at e15.5, e17.5, e19.5 and e21.5 in testes from control and DBP-exposed animals. As previously demonstrated in chapter 3, AR expression was first seen in some, but not all, peritubular myoid cells in control rats at e15.5 (Figure 7.3 A). However, the peritubular myoid cells in e15.5 testes from DBP-exposed animals were all immunonegative (Figure 7.3 B). At e17.5, testes from control animals expressed AR in all peritubular myoid cells (Figure 7.3 C), but in testes from DBP-exposed animals at the same age, only some of the peritubular myoid cells expressed AR and some remained negative (Figure 7.3 D). By e19.5, AR expression was evident in all peritubular myoid cells in testes from control and DBP-exposed animals (Figure 7.3 E and F), and expression remained unchanged at e21.5 (Figure 7.3 G and H).

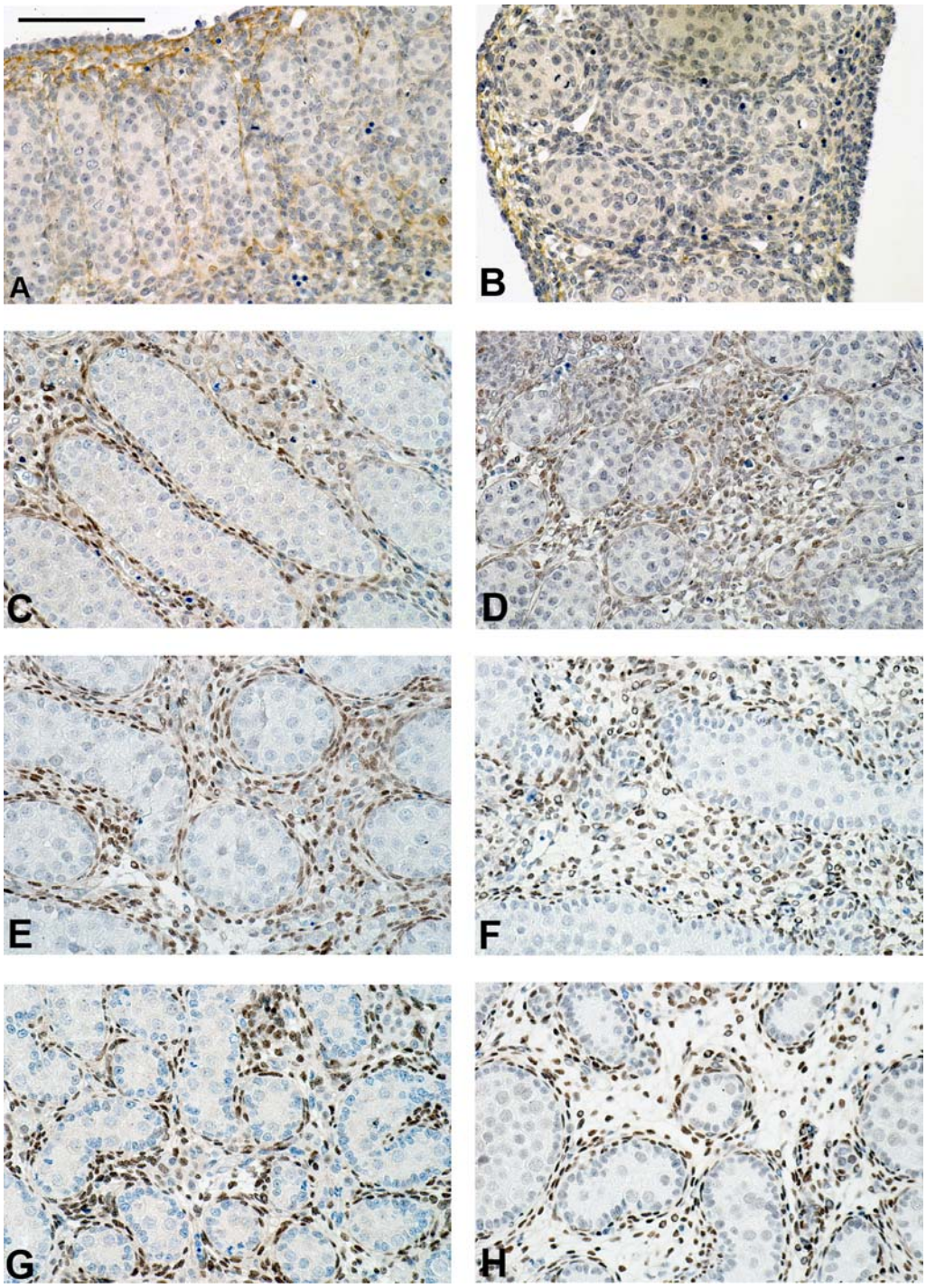

Figure 7.3 Representative photomicrographs of AR immunoexpression at e15.5 (A: control, B: DBP-treated), e17.5 (C: control, D: DBP-treated), e19.5 (E: control, F: DBP-treated) and e21.5 (G: control, H: DBP-treated). Scale bar represents 100μm.

In contrast to the majority of e17.5 testes from DBP-exposed animals, in which AR expression was evident in some but not all peritubular myoid cells, one of the e17.5 DBP-exposed animals had extremely sparse AR expression, and almost all of the peritubular myoid cells were immunonegative (Figure 7.4).

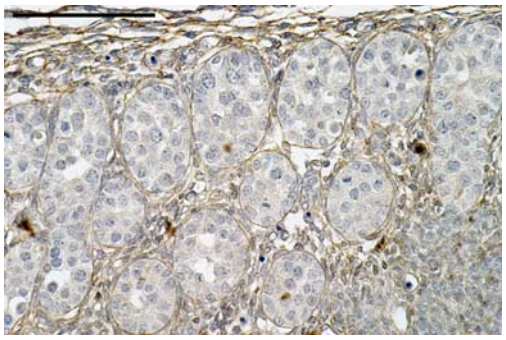

Figure 7.4 AR immunoexpression at e17.5 in the testis of one DBP-exposed animal with marked delay in onset of peritubular myoid cell AR expression. Scale bar represents 100μm.

7.3.3.2 Markers of intermediate filament proteins in peritubular myoid cells

Desmin expression was analysed using immunohistochemistry on testes from control and DBP treated animals at e15.5, e17.5, e19.5 and e21.5. Desmin expression was present in the peritubular myoid cells from e15.5 and expression was unaffected by exposure to DBP (Figure 7.5).

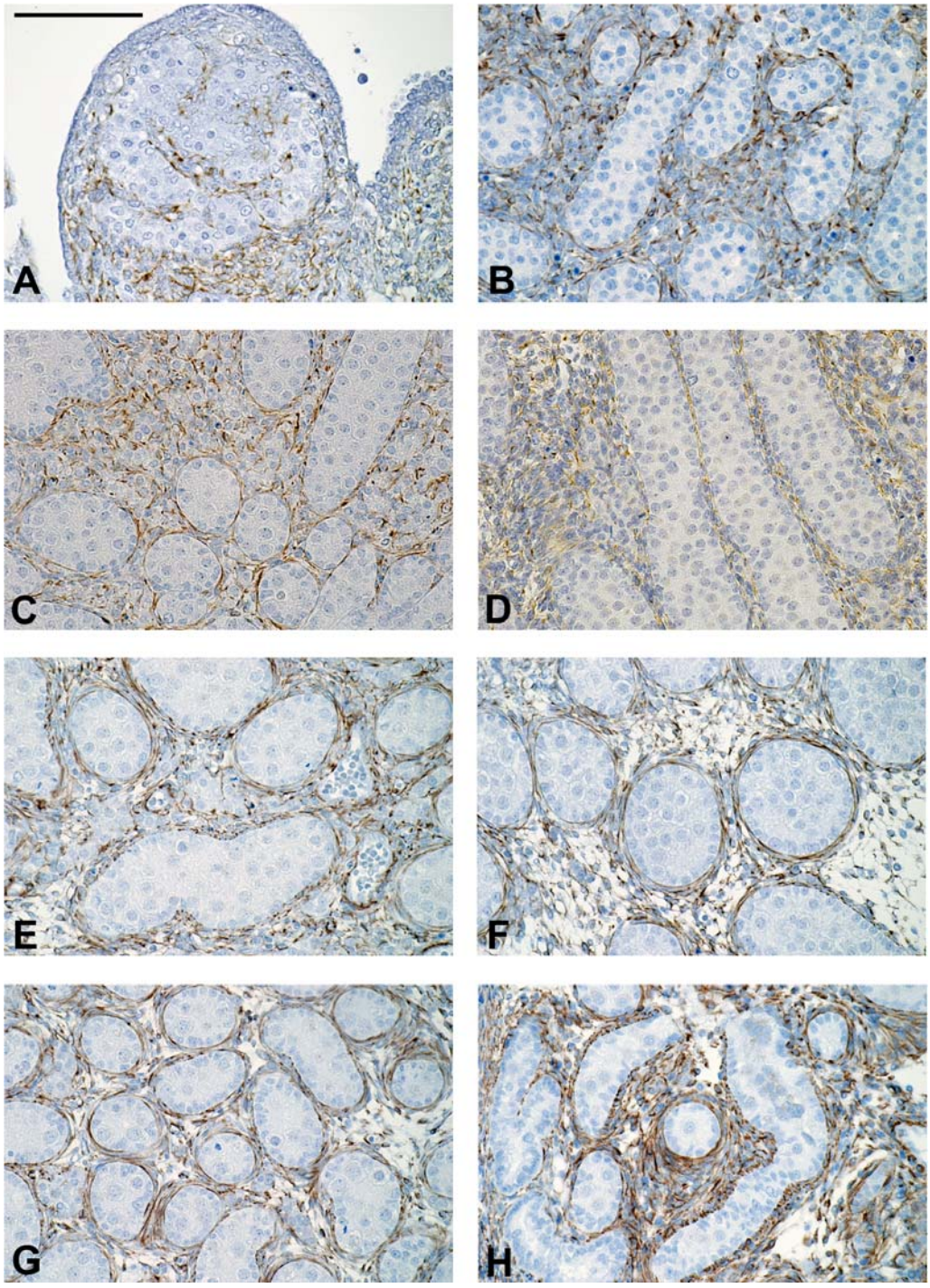

Figure 7.5 Representative photomicrographs of desmin expression at e15.5 (A: control, B: DBP-treated), e17.5 (C: control, D: DBP-treated), e19.5 (E: control, F: DBP-treated) and e21.5 (G: control, H: DBP-treated). Scale bar represents 100μm.

As SMA was one of the first peritubular myoid cell markers investigated, testes taken from e21.5 flutamide and DMBA exposed animals were also analysed, prior to confirmation that neither of these treatments significantly reduced testicular testosterone levels or Sertoli cell number, and would therefore not be expected to induce any change in peritubular myoid cell marker expression. In addition, control animals and DBP exposed animals at e21.5, as well as ARKO and PGK-Cre postnatal day 2 testes were investigated, as previous literature (Schlatt et al., 1993) suggested that SMA is a marker of androgen-induced peritubular myoid cell differentiation. SMA expression appeared to be normal in flutamide and DBP treated animals, but was less intense in testes from DMBA-exposed animals and was completely absent in ARKO testes (Figure 7.6).

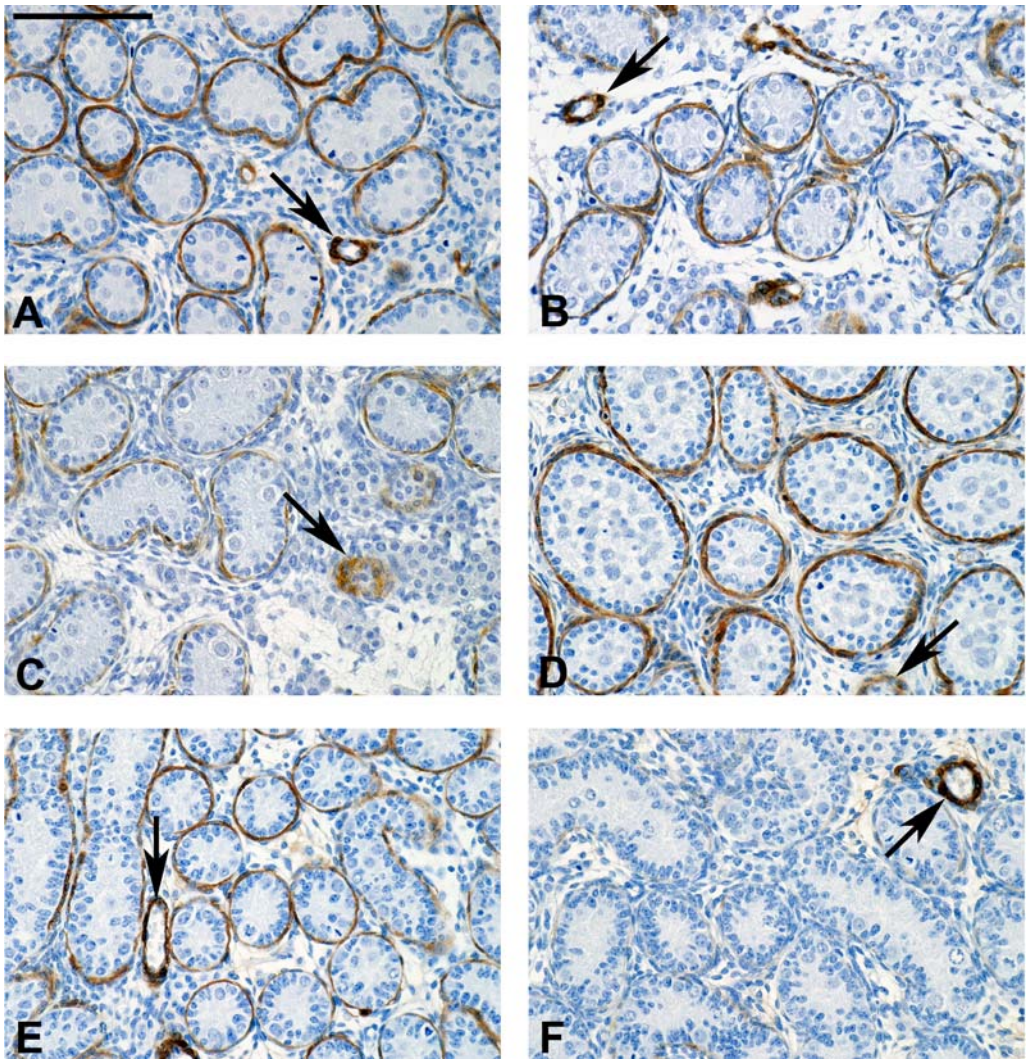

Figure 7.6 SMA expression at e21.5 in testes from A) control, B) flutamide-exposed, C) DMBA-exposed and D) DBP-exposed, animals. The bottom two panels are from postnatal day 2 mice; E) PGK-Cre and F) ARKO. Note the positive SMA immunoexpression in blood vessels (arrows) in all testes including in ARKOs (F). Scale bar represents 100μm.

SMA is not only expressed in the peritubular myoid cells, but also in the contractile cells of blood vessels and in the capsule that encompasses the testis. Consequently, despite observing qualitative changes in SMA expression in testes from DMBA-exposed and ARKO animals, Western blot analysis was not an option for further quantitative analysis, as SMA expression in the blood vessels and capsule would also be included. A new method of stereological analysis was therefore devised to quantify the intensity of SMA staining in the peritubular myoid cells and, in order to eliminate any changes resulting from DAB development, compared to the intensity of staining in the blood vessels (internal control). As was clear from the immunohistochemical analyses, expression of SMA in the ARKO testes was considerably reduced. However, despite appearing less intense in testes from DMBA-exposed animals, SMA expression was unchanged in these as well as in the testes from flutamide-exposed and DBP-exposed animals (Figure 7.7).

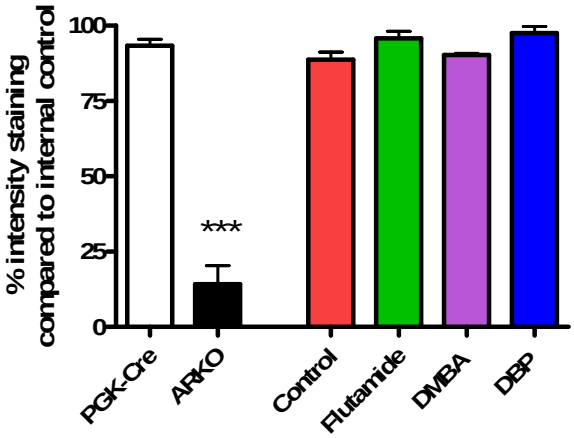

Figure 7.7 Quantification of SMA expression in the peritubular myoid cells of PGK-Cre (n=4) and ARKO (n=4) postnatal day 2 mice and in testes from control (n=4), flutamide- (n=4), DMBA- (n=4) and DBP- (n=4) -exposed animals at e21.5, compared to the intensity of staining measured in the blood vessels in each testis section (internal control). Values are means ± SEM. ***p<0.001 in comparison with PGK-Cre control.

Although no change in SMA expression had been observed at e21.5 in testes from DBP-exposed animals, testes from e15.5, e17.5 and e19.5 control and DBP-exposed animals were also analysed, to determine at what fetal age SMA expression was first seen and whether expression was delayed or changed at earlier fetal ages after DBP exposure. SMA immunoexpression was not evident at e15.5 testes, in either the blood vessels or the peritubular myoid cells. SMA expression was first demonstrated in the peritubular myoid cells (and blood vessels) at e17.5 in control testes, however, expression in testes from DBP-exposed animals at e17.5 was much fainter and staining was not continuous around the whole cord, as was seen in control testes at the same age (Figures 7.8). By e19.5 SMA expression was comparable in testes from both control and DBP-exposed animals (Figure 7.8).

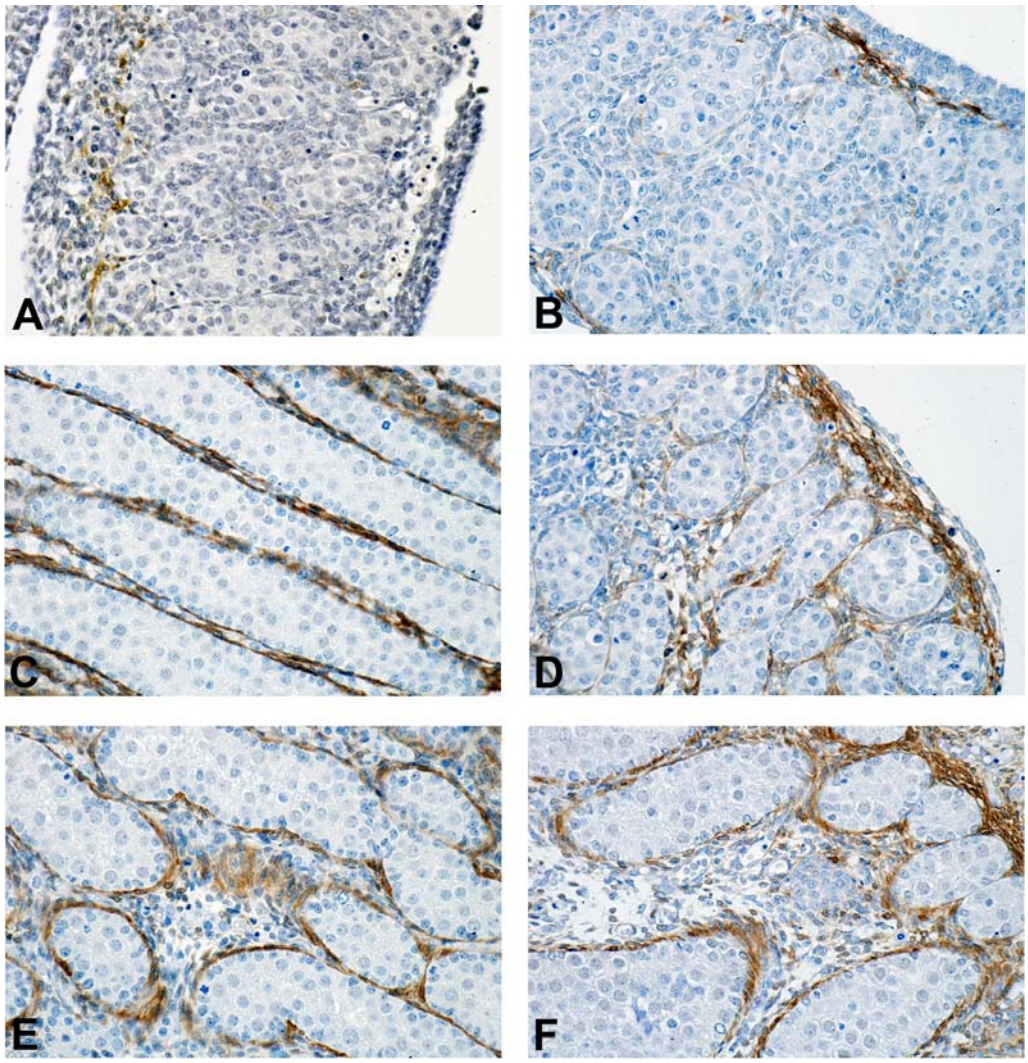

Figure 7.8 Representative photomicrographs of SMA immunoexpression in testes at e15.5 (A: control, B: DBP-treated), e17.5 (C: control, D: DBP-treated) and e19.5 (E: control, F: DBP-treated). Scale bar represents 100μm.

Further examination of SMA staining at e17.5 was performed and images were captured using a higher magnification. This analysis revealed that the majority of peritubular myoid cells did not express SMA at e17.5 after DBP exposure, or expression was weak and this suggested that it was just switching on (Figure 7.9).

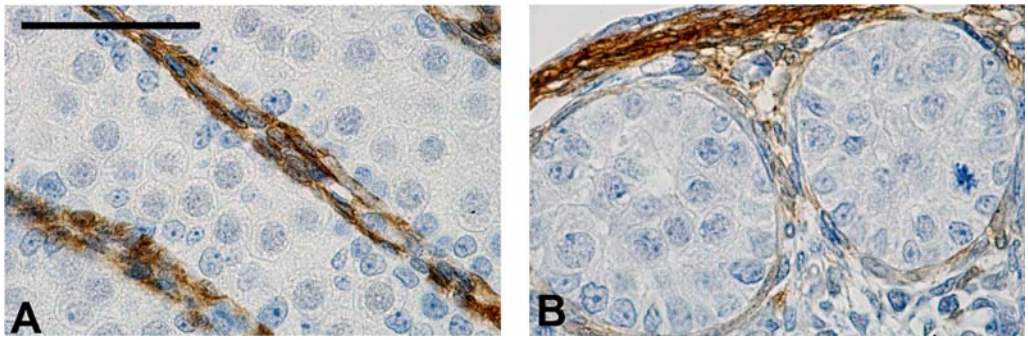

Figure 7.9 SMA immunoexpression at e17.5 in testes from (A) control and (B) DBP-exposed animals. Scale bar represents 50μm.

7.3.3.3 Peritubular myoid precursor cell marker

P75 neurotrophin receptor (P75NTR) immunoexpression was investigated in testes from control and DBP-exposed animals at e15.5, e17.5 and e19.5. There was no obvious, consistent difference in P75NTR expression in the DBP-exposed animals compared to controls at any age. At e15.5, P75 neurotrophin receptor was expressed around the peritubular myoid cells and also around cells in the interstitium (Figure 7.10 A and B). At e17.5 P75NTR expression appeared more restricted to the peritubular myoid cell cytoplasm, although there was still some staining in the interstitium (Figure 7.10 C and D); this pattern of expression persisted at e19.5 (Figure 7.10 D and E).

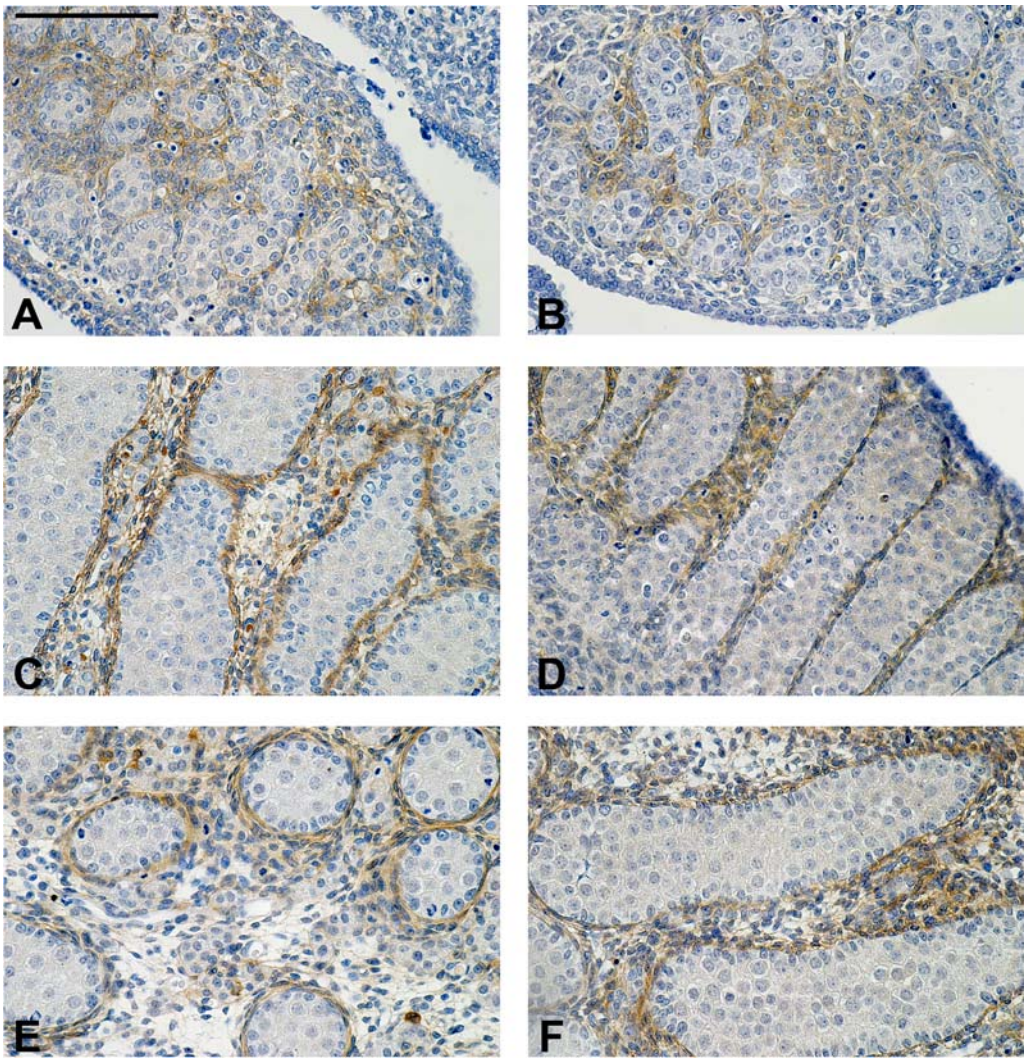

Figure 7.10 Representative photomicrographs of P75NTR immunoexpression at e15.5 (A: control, B: DBP-treated), e17.5 (C: control, D: DBP-treated) and e19.5 (E: control, F: DBP-treated). Scale bar represents 100μm.

7.3.3.4 Extracellular matrix/basal lamina markers

Laminin immunoexpression was investigated at e15.5, e17.5, e19.5 and e21.5 in testes from control and DBP-exposed animals. Laminin staining at e15.5 in control testes was very faint in the extracellular matrix (ECM) of the basement membrane, lying between the peritubular myoid cells and Sertoli cells, and was more prominent in the interstitium (Figure 7.11 A). Laminin was not expressed at e15.5 in testes of DBP-exposed animals (Figure 7.11 B). At e17.5, laminin expression was still very faint, but was present in the ECM of the basement membrane in testes from both control and DBP-exposed animals (Figure 7.11 C and D). At e19.5, laminin staining was more obvious in the basement membrane and less prominent in the interstitium in the control (Figure 7.11 E). Laminin expression in testes of DBP-exposed animals at e19.5 testes was partially present in the basement membrane, but also around the Sertoli cells and between the germ cells and Sertoli cells (Figure 7.11 F). At e21.5, laminin was strongly expressed in the basement membrane of the control testis (Figure 7.11 G), but in testes of DBP-exposed animals at e21.5, expression remained similar to that seen at e19.5, although was perhaps more obvious due to the aggregation of germ cells in the centre of the seminiferous cords (Figure 7.11 H). Overall, laminin immunoexpression in testes from DBP-exposed animals is transiently delayed and instead of being restricted to the basement membrane at e19.5 and e21.5, was also visible around the Sertoli cells.

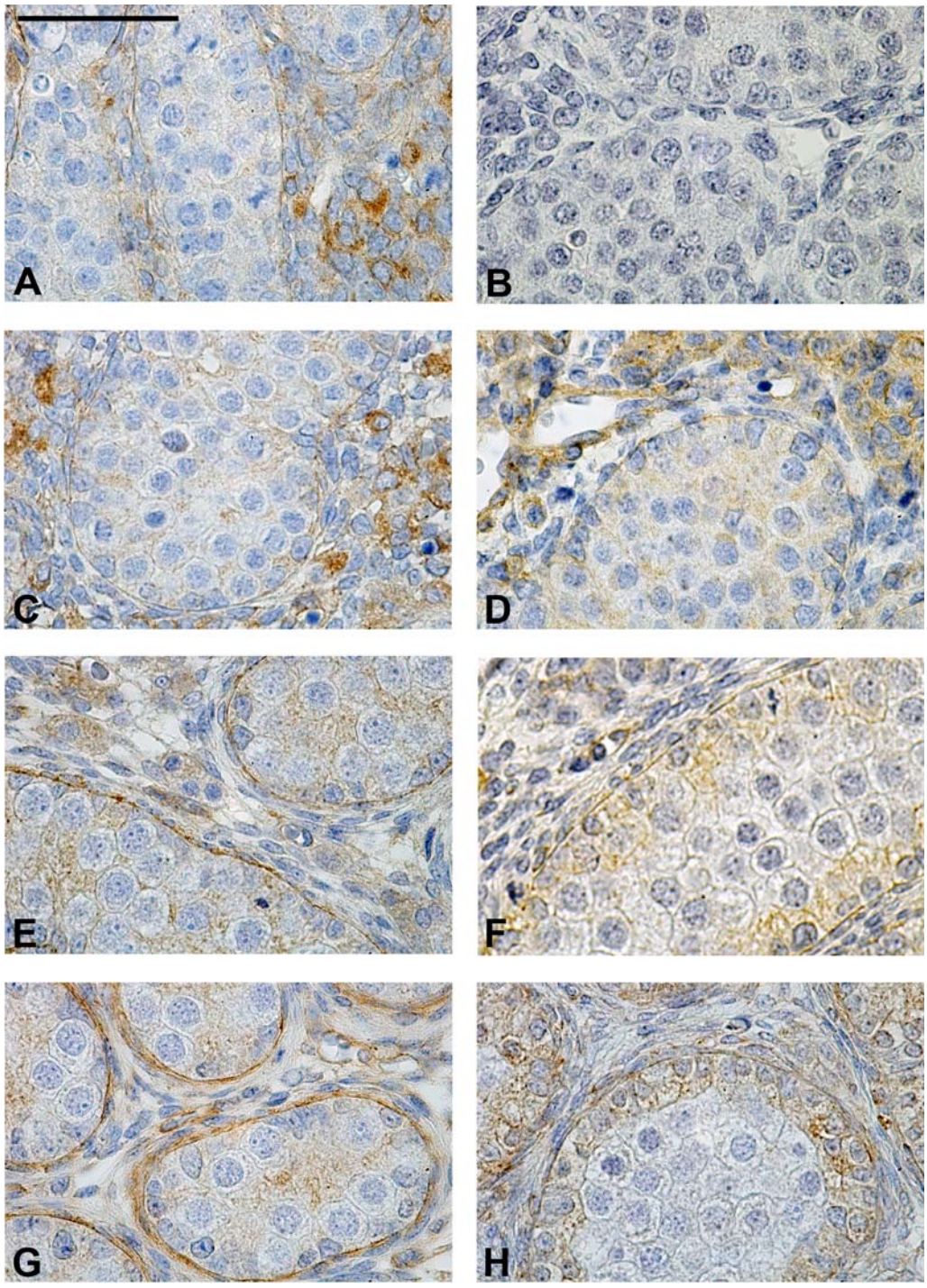

Figure 7.11 Laminin expression at e15.5 (A: control, B: DBP treated), e17.5 (C: control, D: DBP treated), e19.5 (E: control, F: DBP treated) and e21.5 (G: control, H: DBP treated). Scale bar represents 50μm.

Periodic acid schiff (PAS) stains the proteoglycans of the basal lamina (basement membrane), and was used to analyse control and DBP exposed testes at e15.5, e17.5, e19.5 and e21.5, to identify any possible changes in expression after DBP exposure. PAS staining was present in the basal lamina in all ages analysed and no change was observed after DBP exposure (Figure 7.12).

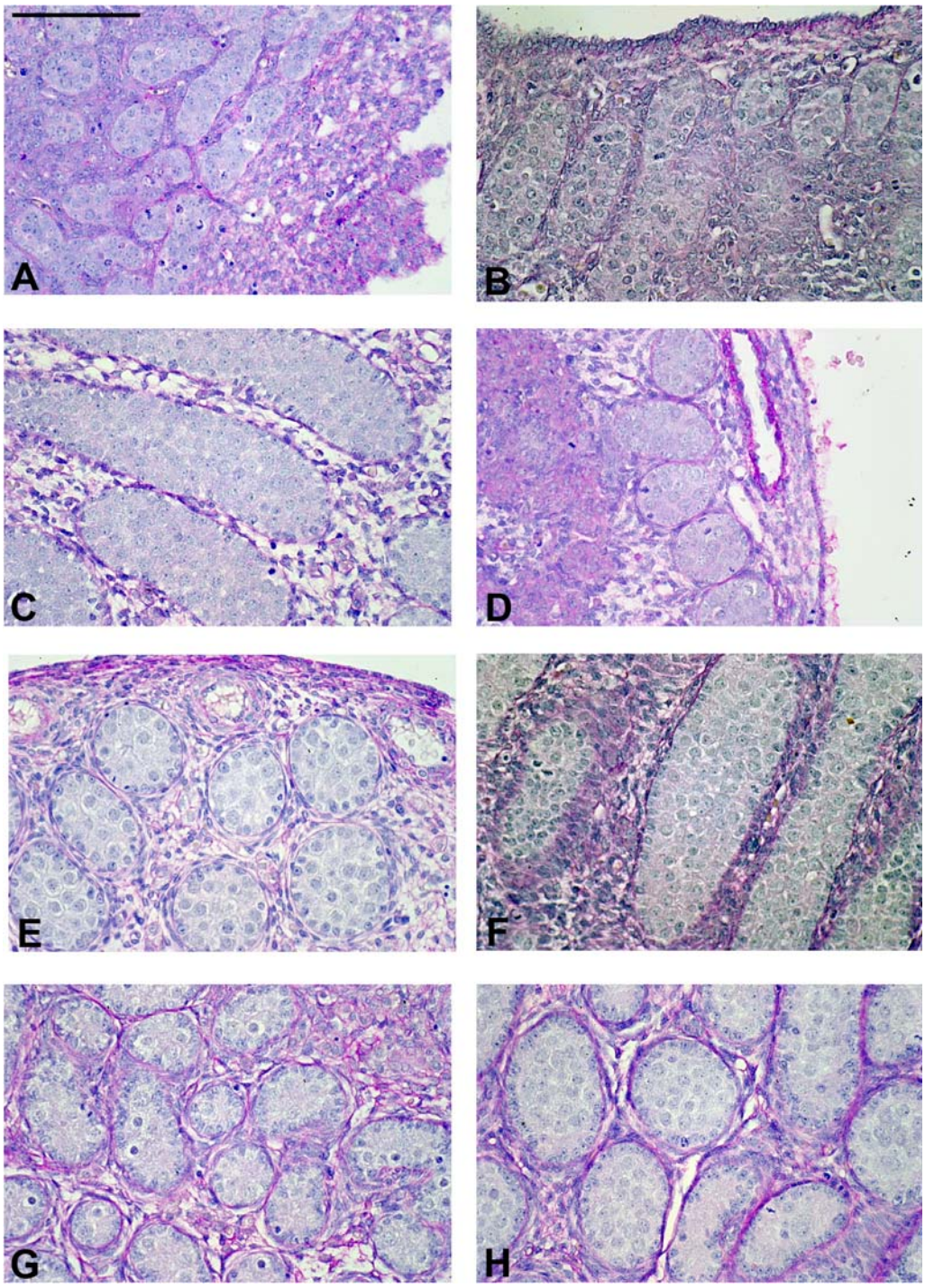

Figure 7.12 PAS staining at e15.5 (A: control, B: DBP treated), e17.5 (C: control, D: DBP treated), e19.5 (E: control, F: DBP treated) and e21.5 (G: control, H: DBP treated). Scale bar represents 100μm.

7.3.3.5 Markers of intermediate filament proteins in Sertoli cells

Vimentin immunoexpression was also investigated in testes of control and DBP-exposed animals at e21.5. DBP exposure did alter the expression pattern of vimentin. In control testes, vimentin staining of the Sertoli cells exhibited finger-like projections which extended in towards the centre of the seminiferous cords, so that staining was evident on either side of the Sertoli cell nucleus as well as at the base (Figure 7.13 A and C). In testes of DBP-exposed animals, vimentin staining was only present at the base of the Sertoli cell and did not extend into the seminiferous cords (Figure 7.13 B and D).

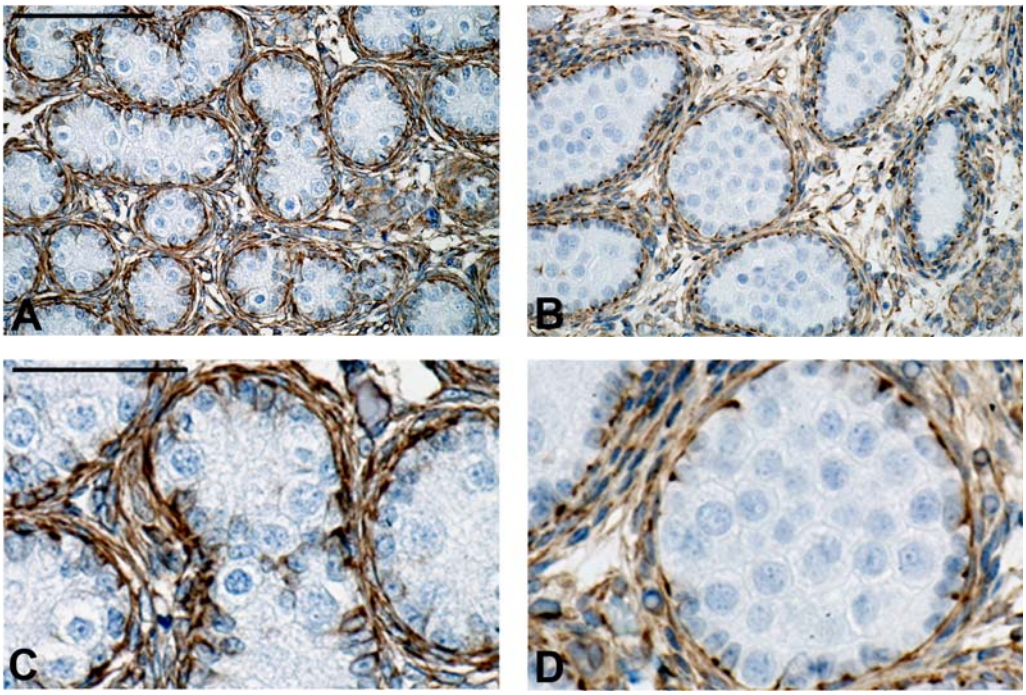

Figure 7.13 Vimentin immunoexpression at e21.5 in testes from control (A and C) and DBP- (B and D) exposed animals. Scale bar in panels A and B represents 100μm, and scale bar in panels C and D represents 50μm.

Nestin immunoexpression was analysed in testes of control and DBP-exposed animals at e15.5, e17.5, e19.5 and e21.5. Nestin expression in controls was observed at the base of the Sertoli cell and around the seminiferous cords as well as in the interstitium from e15.5 to e19.5 (Figure 7.14 A – F). At e21.5 nestin expression was restricted to the base of the Sertoli cell and around the seminiferous cords, with little or no staining in the interstitium (Figure 7.14 G and H). There was no change in the nestin distribution or level of immunexpression after DBP exposure.

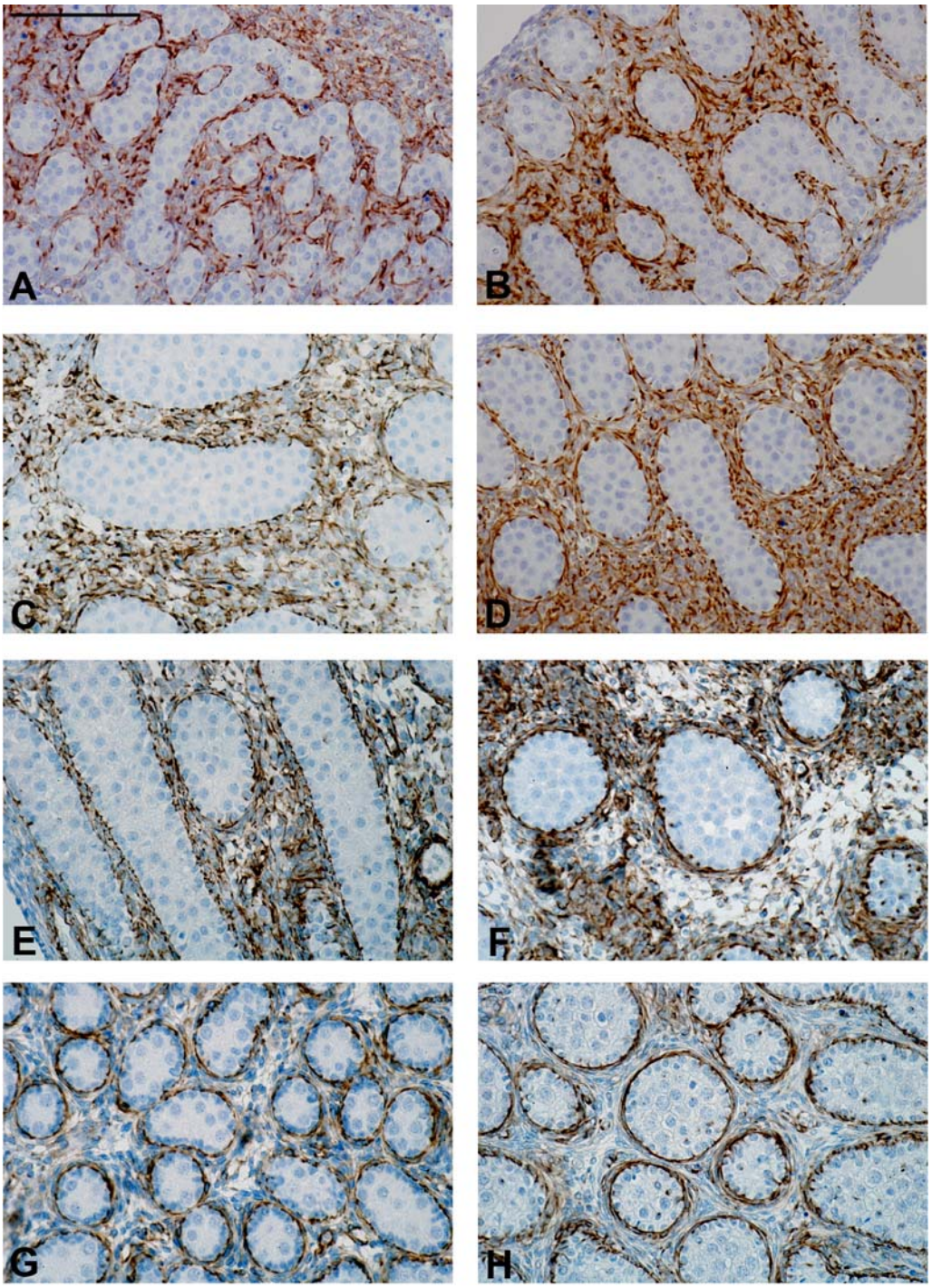

Figure 7.14 Nestin immunoexpression at e15.5 (A: control, B: DBP treated), e17.5 (C: control, D: DBP treated), e19.5 (E: control, F: DBP treated) and e21.5 (G: control, H: DBP treated). Scale bar represents 100μm.

Pancytokeratin detects the expression of cytokeratins 4, 5, 6, 8, 10, 13 and 18, and was investigated in testes from control and DBP-exposed animals at e21.5. Immunostaining demonstrated expression at the base of the Sertoli cells and there was no change in expression after DBP exposure (Figure 7.15).

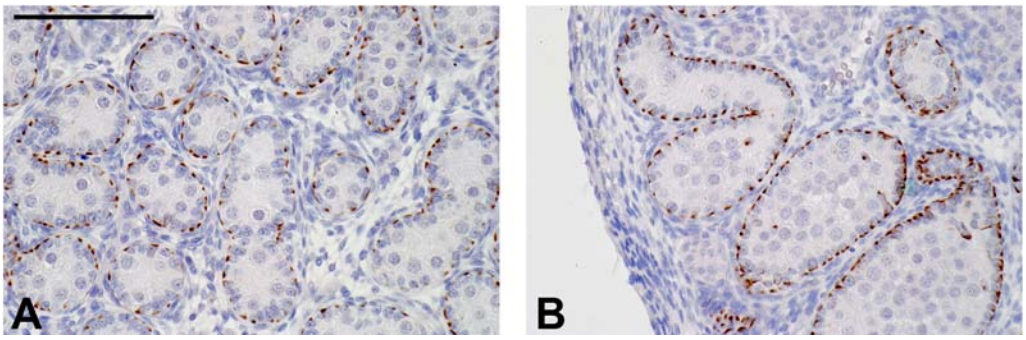

Figure 7.15 Pancytokeratin immunoexpression at e21.5 in testes from (A) control and (B) DBP-exposed animals. Scale bar represents 100μm.

7.3.4 Quantitative analysis of the mRNA expression of various developmental genes in testes of control and DBP-exposed animals

DAX1 mRNA expression levels were analysed in testes from control and DBP-exposed animals at e15.5, e17.5 and e21.5. Expression of DAX1 was low at e15.5 and virtually unaltered after DBP exposure. At e17.5, DAX1 mRNA expression was reduced by 54% in testes of DBP-exposed animals, but this was not statistically significant due to high between-animal variation. At e21.5, DAX1 mRNA expression was significantly reduced (47% reduction) in testes of DBP-exposed animals (Figure 7.16). In controls, DAX1 mRNA expression increased progressively from e15.5 to e21.5 and was 37-fold higher ($p<0.001$) at the latter age than at e15.5. In DBP-exposed animals, the magnitude of increase over the same period was 30-fold ($p<0.01$).

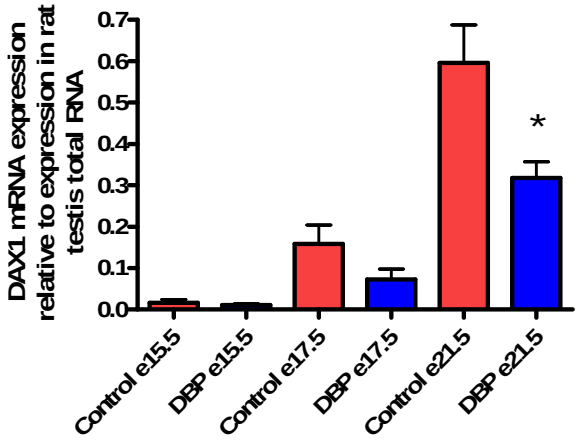

Figure 7.16 Quantitative analysis of DAX1 mRNA levels at e15.5 (control n=4, DBP n=5), e17.5 (control n=5, DBP n=6) and e21.5 (control n=5, DBP n=5) in testes of control and DBP-exposed animals. Values are means ± SEM. *$p<0.05$ in comparison to control value.

It is still unclear where in the fetal testis DAX1 mRNA is expressed (Table 7.2), as several publications have reported various sites of expression. As discussed in chapter 6, immunohistochemistry using a DAX-1 antibody in the present study was performed but was unsuccessful. One recent publication reported DAX1 expression in the fetal Sertoli cells of mice (Meeks et al., 2003). In light of this, DAX1 mRNA expression was corrected at e17.5 and e21.5 for Sertoli cell number. When the reduction in Sertoli cell number seen at e17.5 and e21.5 in DBP-exposed animals (chapter 4) was accounted for, there was no longer a difference in DAX1 mRNA expression between the control and DBP treated groups at either age (Figure 7.17). Based on the similarity in DAX1 mRNA expression per Sertoli cell in control versus DBP-exposed animals at e17.5 and e21.5, these findings would fit with expression of DAX1 being solely or predominantly confined to Sertoli cells.

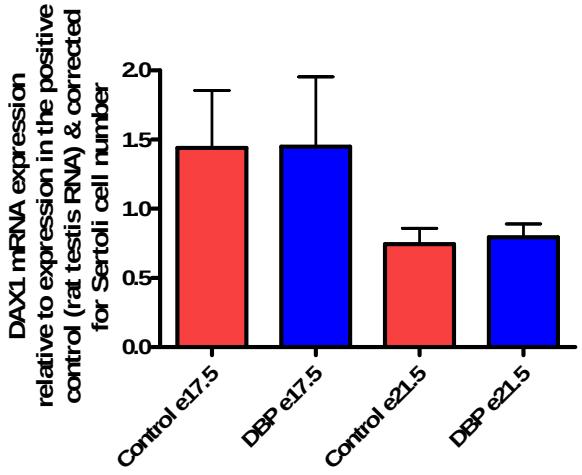

Figure 7.17 Quantitative analysis of DAX1 mRNA levels at e17.5 (control n=4, DBP n=5) and e21.5 (control n=5, DBP n=5) in testes of control and DBP-exposed animals, corrected for Sertoli cell number. Values are means ± SEM.

Dhh mRNA expression levels were investigated in testes of control and DBP-exposed animals at e15.5, e17.5 and e21.5. Dhh mRNA expression levels rose in the control from e15.5 and peaked at e21.5, there being an 8-fold increase between these two ages. Expression levels in testes of DBP-exposed animals at e15.5 and e21.5 appeared to be reduced (Figure 7.18), but neither of these reductions was statistically significant due to high between-animal variation. At e17.5, Dhh mRNA expression levels in testes of DBP-exposed animals appeared higher than in the controls (Figure 7.18), however, due to technical difficulties, only two e17.5 control samples were analysed, so no solid conclusions could be drawn from these results.

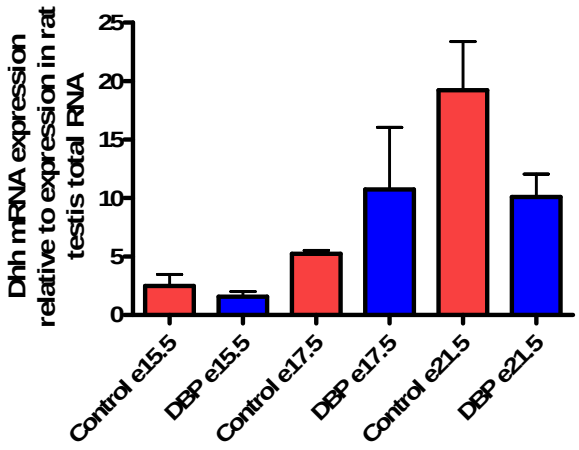

Figure 7.18 Quantitative analysis of Dhh mRNA levels at e15.5 (control n=4, DBP n=5), e17.5 (control n=2, DBP n=6) and e21.5 (control n=4, DBP n=5) in testes of control and DBP-exposed animals. Values are means ± SEM.

DMRT1 mRNA expression levels were analysed at e15.5, e17.5 and e21.5 in testes of control and DBP-exposed animals. Expression was low at e15.5 and was unchanged by DBP exposure. DMRT1 mRNA expression peaked at e17.5 in the control testes and was reduced by 52% in testes of DBP-exposed animals at this age, although this was not statistically significant due to high

between-animal variation. DMRT1 mRNA expression was significantly reduced (50%) in testes of DBP-exposed animals at e21.5 (Figure 7.19)

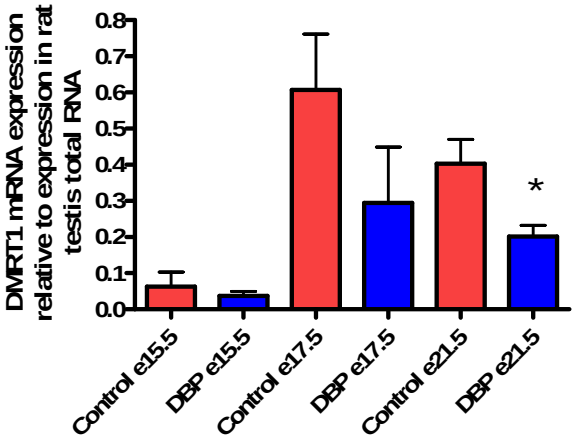

Figure 7.19 Quantitative analysis of DMRT1 mRNA levels at e15.5 (control n=4, DBP=5), e17.5 (control n=5, DBP n=6) and e21.5 Control n=5, DBP n=5) in testes from control and DBP-exposed animals. Values are means ± SEM. *p<0.05 in comparison to control value.

DMRT1 protein expression was investigated using immunohistochemistry at e15.5, e17.5, e195 and e21.5 in testes from control and DBP-exposed animals, to enable a more informed interpretation of the mRNA expression level data. DMRT1 was immunoexpressed in the Sertoli cells and germ cells at e15.5 and e17.5 and there appeared to be no obvious DBP-induced change in the intensity of this expression (Figure 7.20 A – D). At e19.5, only the Sertoli cells were strongly stained for DMRT1 in control testes (Figure 7.20 E), although in testes of DBP-exposed animals, some of the germ cells remained faintly immunopositive for DMRT1 at e19.5 (Figure 7.20 F). At e21.5, DMRT1 immunoexpression was restricted to the Sertoli cells in testes of both control and DBP-exposed animals (Figure 7.20 F and G).

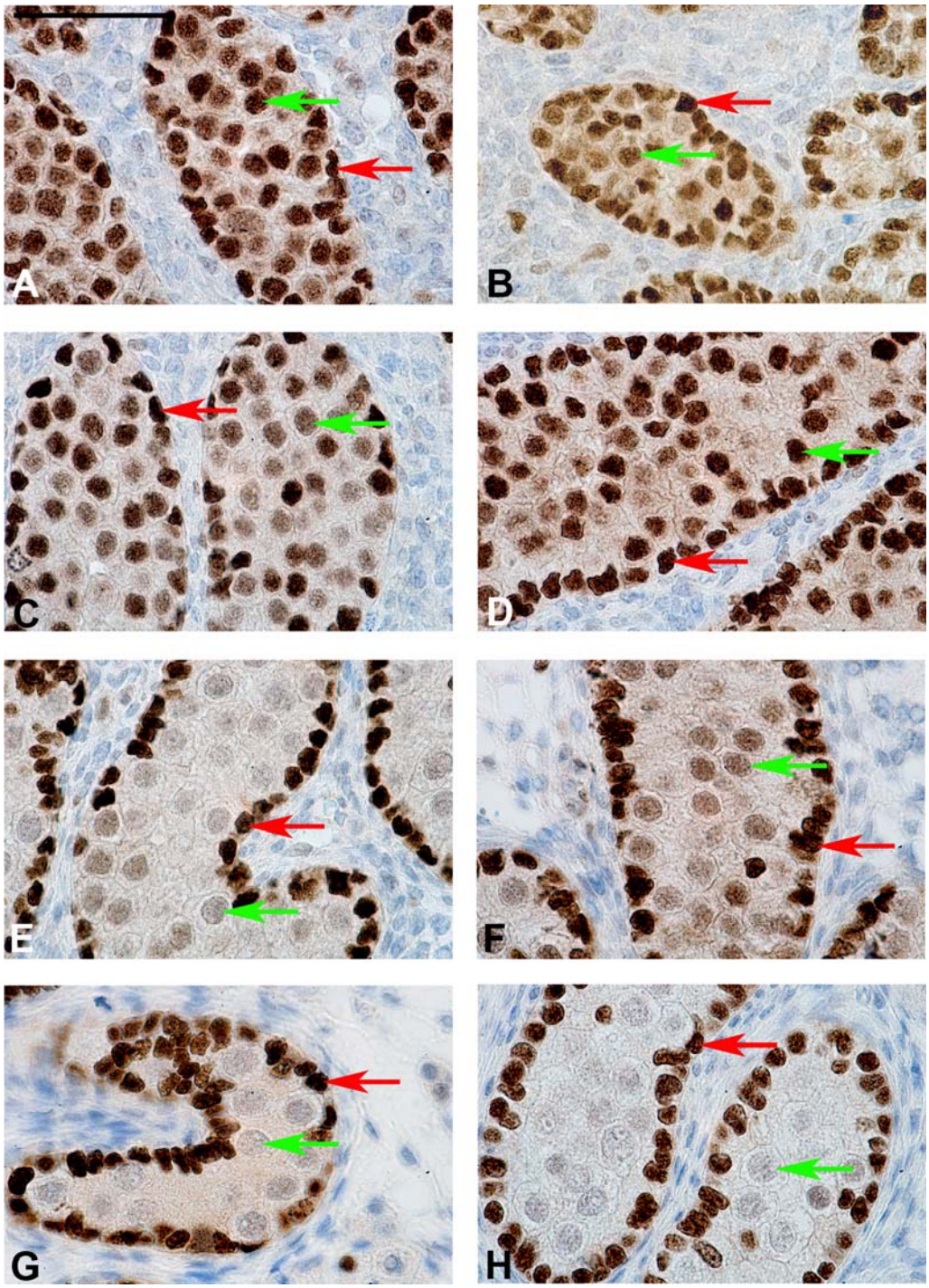

Figure 7.20 Immunoexpression of DMRT1 at e15.5 (A: control, B: DBP treated), e17.5 (C: control, D: DBP treated), e19.5 (E: control, F: DBP treated) and e21.5 (G: control, H: DBP treated). → denote Sertoli cells and → denote germ cells. Scale bar represents 50μm.

Since DMRT1 mRNA was only expressed in Sertoli cells at e21.5, mRNA expression was corrected for Sertoli cell number at this age, to determine whether the reduction in Sertoli cell number in testes from DBP-exposed animals at e21.5 (Figure 7.19) accounted for the reduction seen in mRNA expression. This showed that DMRT1 mRNA expression was the same in control and DBP exposed testes at e21.5 when corrected for Sertoli cell number (Figure 7.21).

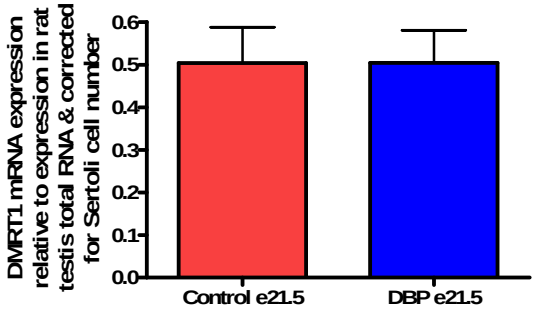

Figure 7.21 Quantitative analysis of DMRT1 mRNA levels at e21.5 (control n= 5, DBP n= 6) in testes from control and DBP-exposed animals after correction for Sertoli cell number. Values are means ± SEM.

FGF9 mRNA expression levels were investigated in testes of control and DBP-exposed animals at e15.5, e17.5 and e21.5. Expression levels were unchanged in DBP exposed animals at e15.5. FGF9 mRNA expression peaked at e17.5 in controls and was reduced by 40% in testes of DBP-exposed animals at this age, although this reduction was not statistically significant due to high between-animal variation. mRNA expression levels for FGF9 fell in controls from e17.5 to e21.5, and at the latter age there was no difference between controls and DBP-exposed animals in the level of (Figure 7.22).

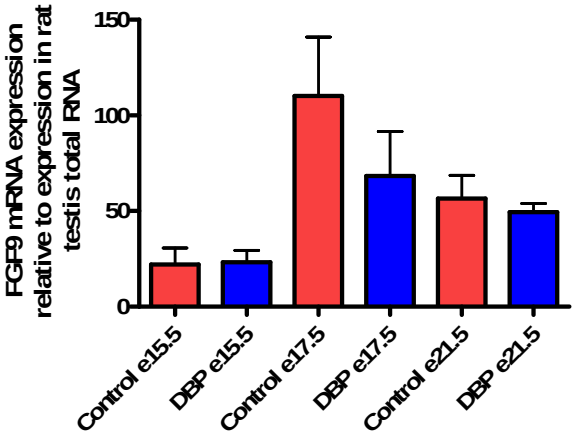

Figure 7.22 Quantitative analysis of FGF9 mRNA levels at e15.5 (control n= 4, DBP n=5), e17.5 (control n=5, DBP n=6) and e21.5 (control n=5, DBP n=5) in testes from control and DBP-exposed animals. Values are means ± SEM.

FGF9 has been reported to be expressed in the fetal Sertoli cells (Kim et al., 2006), so mRNA expression levels were corrected for Sertoli cell number at e17.5 and e21.5. Adjusting for Sertoli cell number resulted in equal mRNA expression levels between the testes from control and DBP-exposed animals (Figure 7.23).

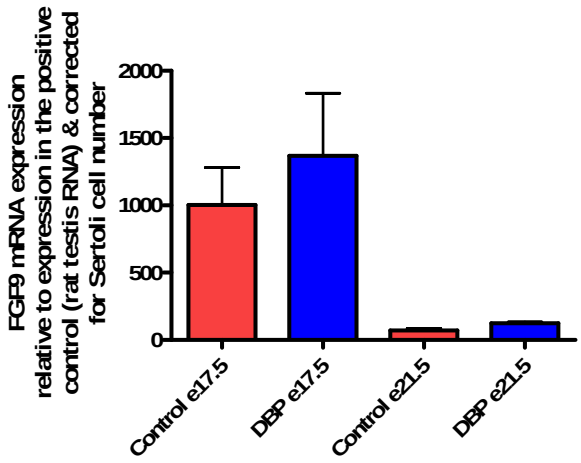

Figure 7.23 Quantitative analysis of FGF9 mRNA levels at e17.5 (control n=5, DBP n=6) and e21.5 (control n=5, DBP n=5) in testes from control and DBP-exposed animals, corrected for Sertoli cell number. Values are means ± SEM.

FMR1 mRNA expression levels were analysed in testes of control and DBP-exposed animals at e15.5, e17.5 and e21.5. FMR1 mRNA levels increased 7-fold from e15.5 to e17.5 in the controls and then remained at the same level of expression at e21.5. At e15.5 in testes of DBP-exposed animals FMR1 mRNA expression was reduced by 41%, by 38% at e17.5 DBP and by 50% at e21.5 (Figure 7.24). However, none of these reductions were statistically significant due to high between-animal variation.

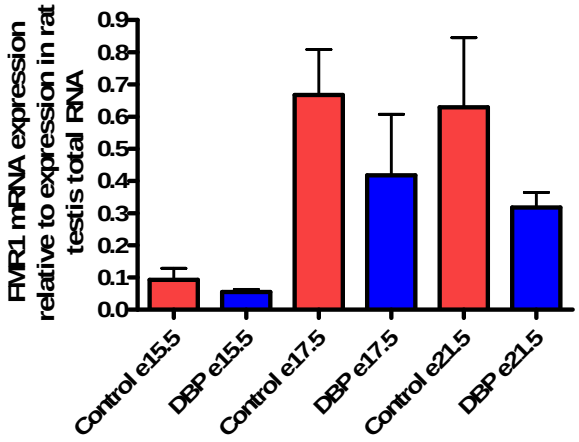

Figure 7.24 Quantitative analysis of FMR1 mRNA at e15.5 (control n= 4, DBP n=5), e17.5 (control n=5, DBP n=6) and e21.5 (control n=5, DBP n=5) in testes from control and DBP-exposed animals. Values are means ± SEM.

7.4 Discussion

7.4.1 Effect of reduced testicular testosterone on peritubular myoid cell number, function and morphology

This section of the chapter sought to identify whether i) androgens are involved in the regulation of peritubular myoid cell proliferation and if peritubular myoid cell numbers are reduced after *in utero* exposure to DBP, and ii) whether reduced testicular testosterone levels, as seen with DBP exposure, resulted in a change or delay in any of the peritubular myoid cell markers investigated. This would potentially offer an explanation as to how

reduced testicular testosterone levels may indirectly affect the Sertoli cells, via the peritubular myoid cells.

In utero exposure to DBP, and hence reduced testicular testosterone levels, did not significantly reduce peritubular myoid cell number at e17.5 or e21.5, suggesting that peritubular myoid cell proliferation is not androgen regulated, and that the reduction seen in Sertoli cell number is not due to a reduction in peritubular myoid cells. Further evidence that the relationship between Sertoli cell number and peritubular myoid cell number is not a simple one, can be seen in the Sertoli cell to peritubular myoid cell ratio, which decreased with age in the controls, but remained low and constant after DBP exposure.

Immunohistochemical analyses of various peritubular myoid cell or peritubular myoid cell related markers in testes of control and DBP-exposed animals, demonstrated that the expression of desmin, P75NTR , PAS, nestin and pancytokeratin was unchanged in response to DBP exposure and reduced levels of testicular testosterone. Expression of AR, SMA, laminin and Vimentin however, were changed after DBP exposure.

Immunoexpression of AR was delayed in testes from DBP-exposed animals, first switching on at e17.5 instead of e15.5 as in controls. It is possible that this delay in expression was due to the reduction in testicular testosterone levels, as it has been previously demonstrated that androgens enhance AR expression (Bentvelsen et al., 1995), and that reduced intratesticular testosterone levels in the adult rat, result in reduced AR expression (Hill et al., 2004). Although the immunoexpression of AR was delayed after DBP exposure, there was no change in the intensity of immunoexpression when it occurred. This is in contrast to a previous publication reporting that AR

protein expression was increased and more intense at gestational days 16, 18 and 21 after DBP exposure (Mylchreest et al., 2002).

SMA has been reported to mark differentiated peritubular myoid cells (Palombi et al., 1992; Paranko and Pelliniemi, 1992; Tung and Fritz, 1990). *In utero* exposure to DBP resulted in a transient delay in peritubular myoid cell differentiation, as demonstrated by delayed expression of SMA. SMA expression was comparable to that in the control by e19.5. This DBP-induced delay in peritubular myoid cell differentiation has been reported before, although peritubular myoid cell differentiation was said to be delayed at e19.5 also in this earlier study (Fisher et al., 2003). The authors interpreted this delay as possibly being due to the DBP-induced reduction in testicular testosterone levels, as it has been suggested previously that peritubular myoid cell differentiation is driven by androgens (Schlatt et al., 1993). Evidence from this thesis, however, demonstrates that peritubular myoid cell differentiation is not androgen dependent, as although peritubular myoid cell differentiation was slightly delayed in ARKO mice, SMA expression is normal by early puberty (unpublished data from our group).

Laminin is a major glycoprotein component of the basement membrane. Although Skinner et al (Skinner et al., 1985) reported that only the Sertoli cells synthesise laminin, other studies have since reported that both the Sertoli cells and peritubular myoid cells synthesise it (Davis et al., 1990; Richardson et al., 1995). Immunoexpression of laminin after *in utero* exposure to DBP was delayed until e17.5, whereas it was present at e15.5 in the control testes, and expression at e19.5 and e21.5 was not restricted to the base of the Sertoli cell, as it was in the controls, but was present around the whole of the Sertoli cell. It has been reported that laminin is required for the migration of

peritubular myoid cells around the Sertoli cells during cord formation and to permit cohesion between the Sertoli cells and peritubular myoid cells (Tung and Fritz, 1994), so this delay in laminin expression may be indicative of a delay in the co-ordination of the Sertoli cells and peritubular myoid cells and thus the early stages of basement membrane formation. It has also been reported that the compaction and polarisation of the Sertoli cell precursors may require laminin at the epithelial-mesenchymal cell interface (Tung and Fritz, 1994). This suggests that the abnormal laminin expression seen in testes of DBP-exposed animals at e19.5 and e21.5 may indicate that the Sertoli cells have not polarised normally, and may also be the reason that they have not polarised.

The accumulation of vimentin filaments in the basal cytoplasm of the Sertoli cells has previously been described as a marker for Sertoli cell polarisation (Kanai et al., 1992). Vimentin expression was only investigated at e21.5 in testes from control and DBP-exposed animals, and vimentin was expressed at the base of the Sertoli cells, implying that they had polarised successfully. However, in DBP-exposed animals, vimentin staining was not seen in the Sertoli cell processes extending toward the apex of the Sertoli cell, as occurred in controls. This DBP-induced alteration in vimentin expression had been reported previously at e21.5 (Kleymenova et al., 2005) and also in mature rat Sertoli cells exposed to di(*n*-pentyl) phthalate (Creasy et al., 1987). It has also been shown that the vimentin cytoskeleton can be altered in adult Sertoli cells by experimentally reducing intratesticular testosterone (Show et al., 2003), which suggests that the change in vimentin expression seen in the testes of DBP-exposed animals at e21.5, may be due to the reduction in testicular testosterone.

Data from this section demonstrates that the DBP-induced reduction in testicular testosterone levels did not result in a reduction in peritubular myoid cell number, suggesting that peritubular myoid cell proliferation is not regulated by androgens and that the reduction seen in Sertoli cell number is not related to a reduction in peritubular myoid cell number. However, reduced testicular testosterone levels may be responsible for the transient delay in peritubular myoid cell differentiation, altered vimentin expression in the Sertoli cell, and the delay in onset of AR expression in peritubular myoid cells. They may also be responsible for the delay and change in laminin expression, although it is possible that these effects are a result of DBP exposure, rather than reduced testiscular testosterone, as preliminary investigations showed no change in laminin expression in ARKO testes.

There are other potential peritubular myoid cell markers that could be investigated to further elucidate the effects of reduced testicular testosterone on the peritubular myoid cell, including collagen type-1, caldesmon 1 and tropomyosin 1 (Jeanes et al., 2005), and further investigation of these markers in the ARKO or *tfm* testes would provide more conclusive evidence that changes seen in marker expression were a result of reduced/ablated testosterone, not of DBP action.

7.4.2 DBP-induced alterations to the mRNA expression levels of developmental genes

The aim of this section of the chapter was to determine whether DBP exposure altered the mRNA expression of a variety of genes known or suspected to be involved in fetal testis development, and if so, whether these alterations could contribute causally to the TDS-like endpoints seen in testes

of DBP-exposed animals. Taqman quantitative RT-PCR was performed on testes from control and DBP-exposed animals at e15.5, e17.5 and e21.5, and five developmental genes were analysed.

DAX1 mRNA expression was shown to be reduced at e17.5 and e21.5 in testes of DBP-exposed animals, although this reduction was only statistically significant at e21.5, due to high between-animal variation in the e17.5 samples. The site of DAX1 mRNA expression remains rather elusive, as a variety of publications have reported expression in different locations, for example in fetal mice Sertoli cells (Meeks et al., 2003) or fetal mice Leydig cells (Ikeda et al., 1996). When DAX1 mRNA levels were corrected for Sertoli cell number, however, the difference between expression levels in testes of controls and DBP-exposed animals at both ages was eliminated, suggesting that DAX1 mRNA is expressed in the Sertoli cells. If this presumption is correct, it suggests that the reduction in DAX1 mRNA expression seen after DBP exposure is in fact an artefact of reduced Sertoli cell number, rather than a real reduction.

Dhh mRNA expression was reduced at e15.5 and e21.5, but increased at e17.5, in testes of DBP-exposed animals. However, none of these changes in Dhh mRNA expression levels were statistically significant and, due to technical difficulties, only two results were obtained for the e17.5 control group, so no firm conclusions can be taken from this data.

DMRT1 protein was investigated by immunohistochemistry and expression was demonstrated in the Sertoli cell and germ cells up to e17.5 in the controls, and then in Sertoli cells only. In testes of DBP-exposed animals at e19.5, it appeared that DMRT1 expression was slightly prolonged in some germ cells, perhaps providing further evidence for a delay in normal germ

cell differentiation in DBP-exposed animals (Ferrara et al., 2006), although the potential mechanisms and consequences of this are unknown and e19.5 testes were not investigated at the mRNA level. DMRT1 mRNA expression was reduced in testes from DBP-exposed animals at all ages, although this was only statistically significant at e21.5. DMRT1 mRNA expression was corrected for Sertoli cell number at e21.5, when it was shown that DMRT1 was expressed only in the Sertoli cells. When DMRT1 mRNA expression levels were corrected for Sertoli cell number, the reduction previously seen as a result of DBP exposure, was removed, again suggesting that the reduction seen in DMRT1 mRNA expression was a consequence of the reduction in Sertoli cell number (and perhaps germ cell number at e17.5), rather than being a real reduction.

FGF9 mRNA expression was unchanged in testes from DBP-exposed animals at e15.5 and e21.5. Expression was reduced at e17.5 in DBP-exposed animals, but this was not statistically significant, due to high between-animal variation. When FGF9 mRNA expression was corrected for Sertoli cell number at e17.5 and e21.5, the expression of FGF9 at e17.5 in testes of DBP-exposed animals increased marginally, suggesting that either FGF9 expression is not restricted to the Sertoli cells alone, or that the reduction seen at e17.5 in testes of DBP-exposed animals is real and may play a role in reduced cell proliferation, as suggested by the literature (Colvin et al., 2001).

Although FMR1 mRNA expression appeared to be reduced at e15.5, e17.5 and e21.5 in testes of DBP-exposed animals compared to control, none of these reductions were statistically significant due to high between-animal variation. Since the inactivation of FMR1 leads to increased Sertoli cell proliferation (Slegtenhorst-Eegdeman et al., 1998), and testes from DBP-

exposed animals demonstrate a reduction in Sertoli cell number, an increase in FMR1 would perhaps have seemed more likely.

Results from Taqman analysis have demonstrated that it is unlikely that any of the genes analysed are involved in the TDS-like endpoints seen after fetal exposure to DBP. They also highlight the importance of recognising changes in cell number when interpreting mRNA expression data, as this can often explain changes in mRNA expression, as demonstrated with DAX1 and DMRT1, the only two genes that showed a statistically significant reduction in mRNA expression after DBP exposure. Consequently these genes require no further investigation, although there are other genes known or suspected to be involved in testis development, for example M33 (Katoh-Fukui et al., 1998), Arx (Kitamura et al., 2002) and Gata4/Fog2 (Tevosian et al., 2002), that would be interesting to investigate, to further explore the possible mechanisms of DBP action on the fetal testis.

8 Final Discussion

The studies in this thesis stem from an interest in TDS and the mechanisms via which it can arise. The aims of this thesis were to establish if reduced androgen levels/action in the fetal rat testis contributes to putative testicular dysgenetic features, namely reduced Sertoli cell number (chapter 4), occurrence of multinucleated gonocytes or abnormal aggregation of fetal Leydig cells, the precursor of focal dysgenesis (chapter 5); these endpoints were also evaluated in mice with inactivation of the androgen receptor (*tfm* or ARKO mice). A further objective was to induce intrauterine growth restriction (dexamethasone; chapter 6), another risk factor for TDS, and determine what effect, if any, this had on the TDS-like endpoints investigated. As androgen action on Sertoli cells is assumed to be mediated indirectly, via the peritubular myoid cells, changes in peritubular myoid cell number and function were investigated in testes with suppressed androgens (chapter 7) to determine whether reduced androgens affect the peritubular myoid cells and their ability to communicate with the Sertoli cells. *In vitro* studies were also used to investigate the role of androgens in Sertoli cell proliferation (chapter 4), and potential non-androgen related mechanisms of DBP action were investigated using Taqman RT-PCR to determine the mRNA expression of key developmental genes after exposure to DBP (chapter 7).

Evidence from *tfm* mice suggests that the suppressed intratesticular testosterone levels associated with TDS may account for subsequent low sperm counts, via a reduction in perinatal Sertoli cell proliferation/number (Johnston et al., 2004). Sertoli cells can only support a finite number of germ cells through spermatogenesis, so Sertoli cell number per testis determines

how many germ cells can be supported, and consequently, the spermatogenic capability of the testis (Sharpe et al., 2003). To investigate the hypothesis that androgens play a role in Sertoli cell proliferation, pregnant rats were exposed to treatments or co-treatments expected to manipulate intratesticular testosterone levels (DBP, TP and dexamethasone; shown to reduce intratesticular testosterone in chapter 6) or action (flutamide, DMBA). Sertoli cell number was also investigated in mice with complete knockout of the androgen receptor (ARKO mice).

Sertoli cell number in the ARKO mice, confirmed the findings of (Johnston et al., 2004), and demonstrated that in the complete absence of androgen action, Sertoli cell number is reduced by ~50%. Similar data has since been published by (Tan et al., 2005). It was already well established that exposure to DBP results in a reduction in intratesticular testosterone (Fisher et al., 2003; Mylchreest et al., 1998; Mylchreest et al., 1999; Mylchreest et al., 2000), and the present data confirmed this, as well as demonstrating that *in utero* exposure to dexamethasone causes a significant reduction in testicular testosterone levels. Administration of DBP as a co-treatment with any of the other treatments investigated in this thesis, led to a greater reduction in intratesticular testosterone levels, and all of the treatments that reduced testicular testosterone levels significantly (DBP ± co-treatments and dexamethasone), also resulted in a significant reduction in Sertoli cell number. *In utero* exposure to TP resulted in a reduction in Sertoli cell number, although testicular testosterone levels were not reduced significantly due to between-animal variation. At a treatment level, this data strongly suggests that androgens play a role in Sertoli cell proliferation, although DBP may also have directly affected the Sertoli cells, as has previously been shown to be the case postnatally (Lloyd and Foster, 1988),

and dexamethasone may affect the Sertoli cells directly by acting on the GR, expressed in the Sertoli cells from e17.5 onwards (chapter 6). In order to confirm unequivocally, that androgen action is required for normal fetal Sertoli cell proliferation, Sertoli cell proliferation/number would need to be investigated following *in utero* exposure to a treatment that is likely to have no other direct effect on the Sertoli cells, for example ketoconazole, an anti-fungal drug, that in mammals inhibits P450 dependent enzymes (Higashi et al., 1987), thus inhibiting steroidogenesis (section 1.4). Alternatively, further investigation of the dexamethasone exposed animals, at a time point prior to GR expression in the Sertoli cells (e17.5), may facilitate a definitive conclusion on the role of androgens in fetal Sertoli cell proliferation.

Investigation into Sertoli cell proliferation/number after exposure to various dose levels of DBP also suggested a threshold level of testicular testosterone that was required for normal Sertoli cell proliferation, for although exposure to 100mg/kg DBP resulted in a significant reduction in testicular testosterone levels, it did not cause a reduction in Sertoli cell proliferation/number. To further investigate the critical intratesticular threshold level, the impact of dose levels of DBP between 100mg/kg and 500mg/kg on testicular testosterone and Sertoli cell number would need to be investigated.

In utero exposure to flutamide and DMBA, expected to block androgen action, had no effect on Sertoli cell number. DMBA is a polycyclic aromatic hydrocarbon (PAH) that acts through the Aryl hydrocarbon receptor (AhR), and it had been reported that PAHs act as anti-androgens *in vitro* in transfected cells (Vinggaard et al., 2000), and that activation of the AhR is thought to antagonize androgen action via interaction with the AR (Kizu et al., 2003). It may be that the dose of DMBA was too low, but other published

data have demonstrated that *in utero* exposure to 2,3,7,8-Tetracholrodibenzo-p-dioxin (TCDD), which like DMBA acts through the AhR, does not impair the AR pathway (Ko et al., 2004), suggesting that the low sperm counts that occur as a result of maternal smoking (Jensen et al., 2004; Storgaard et al., 2003), which are thought to result from a reduction in Sertoli cell number, are not the result of exposure to DMBA antagonism of androgen action on Sertoli cells in fetal life. Flutamide exposure both *in utero* and *in vitro* had no effect on Sertoli cell proliferation (chapter 4). It seems most likely that the levels of testosterone within the testis are too high for flutamide to effectively antagonize them, which supports current literature demonstrating that the same dose of flutamide used for the present studies failed to induce regression of the Wolffian duct in the exposed males (Welsh et al., 2006), consistent with the higher local testosterone levels in this duct compared with at peripheral sites (e.g. prostate, perineum). The dose of flutamide administered *in utero* cannot be further increased due to maternal toxicity, but the most efficient way of investigating this treatment further would be to expand on the *in vitro* studies performed in this thesis; increasing the dose of hydroxyflutamide and increasing the age range of testis explants investigated. It would also be interesting to develop the *in vitro* studies using cyclopamine, which showed that testosterone production was reduced concurrent with reduced Sertoli cell proliferation indices. Investigation of the effect of cyclopamine both at earlier ages *in vitro* and also *in utero*, may offer further clarification of the role that Dhh signalling plays in cord formation, Leydig cell differentiation and consequently Sertoli cell proliferation.

When the studies for this thesis were conceived, it was already well established that Sertoli cells do not express androgen receptors in fetal life (Williams et al., 2001), so it was hypothesised that any androgen effects on

Sertoli cell number occur indirectly, via the peritubular myoid cells (Sharpe, 2006). The literature indicated that peritubular myoid cells do express AR during fetal life (Sharpe, 2005; Tan et al., 2005) and chapter 3) and peritubular myoid cells have been shown previously to communicate with the Sertoli cells, in culture (Buzzard et al., 2003a; Skinner and Fritz, 1985b) and during cord formation, when both cell types contribute to the formation of the basement membrane (Skinner et al., 1985; Tung, 1984). Previous reports of peritubular myoid cell proliferation showed high levels of proliferation during late fetal life, which declined after birth (Palombi et al., 1992). This data was confirmed by the present studies and suggested that peritubular myoid cell proliferation may also be regulated by androgens as their proliferative period coincided with the fetal surge of testosterone. Exposure to DBP however, did not alter peritubular myoid cell proliferation or number (chapter 7). In order to investigate the role that the peritubular myoid cells may play in mediating androgen action on the fetal Sertoli cells, immunohistochemical markers of the peritubular myoid cell, peritubular myoid precursor cells or components of the basement membrane (laid down by both the peritubular myoid cells and Sertoli cells), were explored at various fetal ages, to determine whether reduced testicular testosterone or DBP exposure caused a change or delay of expression, indicating a change in peritubular myoid cell morphology or function. AR and SMA immunoexpression was delayed after *in utero* exposure to DBP (chapter 7), and indicates that the suppressed level of androgens have caused the down-regulation of the AR (Bentvelsen et al., 1995) and that, as previously indicated in the literature, peritubular myoid cell differentiation has also been delayed (Fisher et al., 2003). The literature also suggested that peritubular myoid cell differentiation is driven by androgens (Schlatt et al.,

1993). Evidence from this thesis, however, demonstrated that peritubular myoid cell differentiation is not androgen dependent, as although peritubular myoid cell differentiation was slightly delayed in ARKO mice, SMA expression is normal by early puberty in these animals (unpublished data from our group). It would be interesting to conduct a more detailed investigation into the delay of SMA immunoexpression at the various fetal ages, using the novel quantification system designed during the present studies.

Markers of Sertoli cell intermediate filament proteins were also investigated to determine whether reduced testosterone or DBP exposure altered the structure of the Sertoli cells. The immunoexpression of both laminin (in addition to being delayed) and vimentin suggested that the Sertoli cells may not have polarised completely in the androgen depleted testicular environment (chapter 7). The immunoexpression pattern of vimentin, demonstrated in the present studies, confirms previous reports that DBP alters vimentin expression at e21.5 (Kleymenova et al., 2005) and also in mature rat Sertoli cells exposed to di(*n*-pentyl) phthalate (Creasy et al., 1987). It has also been reported that the vimentin cytoskeleton can be altered in adult Sertoli cells by experimentally reducing intratesticular testosterone (Show et al., 2003). Further investigation into whether Sertoli cell polarisation is impeded by suppressed androgen levels or DBP exposure would be beneficial, as it is possible that failure of the Sertoli cells to polarise results in the aggregation of germ cells identified in DBP-exposed animals. If the apical surface of the Sertoli cell 'reads' like the lateral surface, then the germ cells may have no reason to migrate to the basement membrane. Sertoli cell polarisation could be investigated by looking at the distribution of microtubules, as Sertoli cells are proposed to conform to the typical

arrangement of polarised epithelial cells, whereby microtubules are orientated parallel to the long axis of the cell (Schroer and Sheetz, 1991). The distribution of golgi apparatus within the cell is also reported to be indicative of polarisation and in polarised Sertoli cells produces discrete ring patterns that cap the apical portion of the Sertoli cell and extend down the lateral sides of the nucleus (Redenbach et al., 1995).

Evidence from the DBP-treated rat model for TDS suggests that reduced androgen action may also play a role in testicular dysgenesis (Fisher et al., 2003; Mahood et al., 2005; Mahood et al., 2006; Parks et al., 2000; Shultz et al., 2001) as in patients with CAIS, in whom focal areas of testicular dysgenesis have been reported (Hannema et al., 2006). In addition to this, IUGR has also been reported as a risk factor for human TDS (Sharpe and Skakkebaek, 2003; Skakkebaek et al., 2001), so the effects of both impaired androgen levels/action and IUGR were investigated using the treatments discussed above and analysing fetal TDS-like endpoints seen in the DBP rat model; the occurrence of multinucleated gonocytes and large Leydig cell aggregations (Fisher et al., 2003; Mahood et al., 2005; Mahood et al., 2006), shown to be the precursors for focal dysgenesis in the postnatal and adult rat (chapter 7). The treatment administered to induce IUGR, dexamethasone, did not cause a significant reduction in fetal body weight, perhaps due to the low 'n' numbers used in the present study compared to those that report IUGR. The present study consisted of bodyweights from seven animals, from five litters, whereas a previous publication which reported a significant reduction ($p<0.05$) in bodyweight, had an 'n' of 74 (Drake et al., 2005). *In utero* exposure to TP, however, did result in IUGR, as has been previously reported (Fritz et al., 1984; Manikkam et al., 2004). The present studies demonstrated that

reduced bodyweight did not contribute to TDS-like endpoints or a reduction in Sertoli cell number.

Despite not causing IUGR, *in utero* exposure to dexamethasone did induce a significant reduction in testicular testosterone levels, as discussed previously, and this treatment, along with the analysis of *tfm* and ARKO mice, strongly suggests that the dysgenetic endpoints assessed are the result of DBP-exposure rather than being the result of suppressed androgen levels/action. Consistent with this conclusion, data has just been published demonstrating that *in utero* exposure of mice to DBP does not cause a reduction in testicular testosterone, but still induces formation of multinucleated gonocytes and impaired seminiferous cord formation (specifically increased cord diameter indicative of central aggregation of the germ cells; (Gaido et al., 2007).

Having demonstrated that the TDS-like endpoints exhibited by DBP-exposed animals are not the result of reduced androgen production, further investigation into the non-androgen related mechanisms by which DBP may exert its effects were analysed. A number of genes known or suspected to be involved in fetal testis development, and/or implicated in testicular dysgenesis in knockout studies were selected from the literature and mRNA expression levels were investigated at different fetal ages in testes from control and DBP-exposed animals. mRNA expression levels of DAX1 and DMRT1 were significantly reduced at e21.5 and FGF9 was considerably (although not significantly) reduced at e17.5. The literature suggested that all three of these genes were, or could be, expressed in the Sertoli cells, and when mRNA expression levels were corrected for the reduction in Sertoli cell number caused by DBP exposure, the differences in mRNA expression levels did not persist. Not only does this suggest that these genes are not involved

in the DBP-induced effects on the fetal testis, it also reiterates the importance of taking reduction in cell number into account when analysing mRNA expression data, otherwise completely misleading conclusions could have been reached. Further investigation into other genes, implicated by the literature as having a possible role in fetal testis development, e.g. M33 (Katoh-Fukui et al., 1998) and Arx (Kitamura et al., 2002) may provide more insight into non-androgen mediated DBP mechanisms, but must be analysed with cell number in mind.

This thesis has provided evidence to support the view that suppressed androgen levels/action in perinatal life could lead to subnormal sperm production levels in adulthood, via a reduction in Sertoli cell proliferation/number. However, it still needs to be shown whether the DBP-induced deficit in Sertoli cell number at birth can be compensated for postnatally, and work in our group suggests that compensation does occur (Hutchison et al., 2007). Whether continued exposure to DBP postnatally would result in continued, and permanent suppression of Sertoli cell number seems likely based on the present observations, but has not yet been investigated.

Suppressed androgen levels/action do not induce the TDS-like dysgenetic endpoints investigated in this thesis, i.e. multinucleated gonocytes and large Leydig cell clusters, and IUGR, despite being a risk factor for human TDS, does not cause or exacerbate any of the TDS-like endpoints investigated in the rat animal model for TDS.

Although *tfm* and ARKO mice both exhibit significantly reduced Sertoli cell numbers at birth (Johnston et al., 2004)chapter 3), they still have approximately half of the normal configuration of Sertoli cells, suggesting

that in addition to androgen action, and potential FSH action during the last few days of gestation, other factors are at work, ensuring that Sertoli cells are proliferating, albeit at a reduced rate. Further investigation into potential factors and mechanisms would advance current knowledge in this area. Analysis of *tfm* and ARKO testes in this thesis demonstrated an absence of focal dysgenesis, however, human CAIS patients have been reported to present with focal dysgenesis (Hannema et al., 2006), which suggests potential species differences. A possible explanation as to why human CAIS patients develop focal dysgenesis when ARKO and *tfm* mice do not may be due to the speed at which testes develop in each species. Human testes may take longer to develop and as a consequence steroidogenesis, which occurs fairly early on in testes development, may switch on before the testis is fully formed, thereby creating a window in which androgens, or a lack of androgens may perturb normal testis formation/development. Testis formation in the mouse occurs more rapidly, so there is less opportunity for androgens to play a role. Irrespective of why ARKO and *tfm* mice do not develop focal dysgenesis, this difference between mice and humans highlights the limitations of using animal models, which are not true models for a human disorder.

The studies in this thesis have focused on the role of androgens in perinatal Sertoli cell proliferation/number, and have demonstrated that reduced testicular testosterone is concurrent with reduced Sertoli cell number. Any effects that androgens have on the Sertoli cell however are known to be indirect, since fetal Sertoli cells do not express AR. The indirect mechanism via which androgens act on the Sertoli cells are therefore a priority to further understand the role of androgens in Sertoli cell proliferation. Although the studies in this thesis have not demonstrated any dramatic effects on

peritubular myoid cells, evidence suggests that these are the most likely cell type to mediate androgen action on the Sertoli cells. Recent evidence has demonstrated several genes (*Dusp6*, *Egr1*, *Fos* and *Thbs1*) that are induced within the rat peritubular myoid cells within one hour of exposure to 500mg/kg DBP at e19.5, and the authors suggest that the peritubular myoid cells (and unknown interstitial cells that also expressed *Egr1* and *Fos*) may be the proximal target cells of phthalate exposure (Johnson et al., 2007). Further investigation at earlier fetal ages may reveal a role in the delays seen in testis development, especially the delay in peritubular myoid cell differentiation. The peritubular myoid cell specific AR knockout mice may also provide a useful tool for further investigation into the role that peritubular myoid cells play in mediating androgen action. So far data regarding testis development in these mice has been limited, although defects in Sertoli cell functional marker genes have been reported, as have a ~24% reduction in testis size, a significant reduction in germ cell number and subnormal sperm counts at 14 weeks of age (Zhang et al., 2006). These data may indicate that Sertoli cell number is also reduced, although further investigation is required. The most useful method of investigating the role of peritubular myoid cells in mediating androgen action on the Sertoli cells is likely to be through the use of testis explant cultures. Identification of a time frame in which a reduction in Sertoli cell number/proliferation can be induced, however, is essential to the viability of this system. Explants studies in this thesis focussed on e19.5 testes, so earlier ages need to be investigated. Data from this thesis also suggests that peritubular myoid cell number is not likely to change in testes with suppressed androgen levels/action, so effects may be subtle and it is possible that other factors may compensate.

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